

CONTRACEPTIVE ANTIBODY VACCINES

1. FIELD OF THE INVENTION

5 The present invention relates to modified antibodies, and vaccine compositions thereof, that have one or more complementary determining regions that contain portions of sperm antigens, in which modified antibodies one or more variable region cysteine residues that form intrachain disulfide bonds have been replaced with amino acid residues that do not contain a sulfhydryl group and, therefore, do not form disulfide bonds. The present
10 invention also relates to use of the vaccine compositions of the invention as a contraceptive.

2. BACKGROUND OF THE INVENTION

2.1. IMMUNOGLOBULIN STRUCTURE

The basic unit of immunoglobulin structure is a complex of four polypeptides --
15 two identical low molecular weight or "light" chains and two identical high molecular weight or "heavy" chains, linked together by both noncovalent associations and by disulfide bonds. Each light and heavy chain of an antibody has a variable region at its amino terminus and a constant domain at its carboxyl terminus (Figure 1). The variable regions are distinct for each antibody and contain the antibody antigen binding site. Each variable
20 domain is comprised of four relatively conserved framework regions and three regions of sequence hypervariability termed complementarity determining regions or CDRs (Figure 2). For the most part, it is the CDRs that form the antigen binding site and confer antigen specificity. The constant regions are more highly conserved than the variable domains, with slight variations due to haplotypic differences.

25 Based on their amino acid sequences, light chains are classified as either kappa or lambda. The constant region heavy chains are composed of multiple domains (CH1, CH2, CH3...CHx), the number depending upon the particular antibody class. The CH1 region is separated from the CH2 region by a hinge region which allows flexibility in the antibody. The variable region of each light chain aligns with the variable region of each heavy chain,
30 and the constant region of each light chain aligns with the first constant region of each heavy chain. The CH2-CHx domains of the constant region of a heavy chain form an "Fc region" which is responsible for the effector functions of the immunoglobulin molecule, such as complement binding and binding to the Fc receptors expressed by lymphocytes, granulocytes, monocyte lineage cells, killer cells, mast cells and other immune effector
35 cells.

As seen in Figure 3, the light and heavy chains of an IgG molecule form the variable region domain and the constant region domain. Each domain is composed of a sandwich of two parallel extended protein layers of about 100 amino acids in length which are connected by a single disulfide bond (See Roitt et al., Immunology, 3rd Edition, London; Mosby, 1993, p4.4 (Figure 3)). Each of the two extended protein layers of the domain, in turn, contains two "anti-parallel" adjacent strands which adopt a beta-sheet conformation. (See, e.g., Stryer, 1975, Biochemistry, WH Freeman and Co., p. 950). Each of the domains has a similar three-dimensional structure based on the immunoglobulin fold.

10 2.2. IMMUNOTHERAPY AND ANTI-IDIOTYPE ANTIBODIES

In modern medicine, immunotherapy or vaccination has virtually eradicated diseases such as polio, tetanus, tuberculosis, chicken pox, measles, hepatitis, etc. The approach using vaccinations has exploited the ability of the immune system to prevent infectious diseases.

15 Use of immunotherapy has also been explored for cancer therapy. The era of tumor immunology began with experiments by Prehn and Main, who showed that antigens on the methylcholanthrene (MCA)-induced sarcomas were tumor specific in that transplantation assays could not detect these antigens in normal tissue of the mice (Prehn et al., 1957, J. Natl. Cancer Inst. 18:79-778). This notion was confirmed by further experiments demonstrating that tumor specific resistance against MCA-induced tumors could be elicited in the autochthonous host, that is, the mouse in which the tumor originated (Klein et al., 20:151-1572).

There are many reasons why immunotherapy is desired for use in cancer patients. First, if cancer patients are immunosuppressed in surgery, with anesthesia and subsequent chemotherapy, it may worsen the immunosuppression, then with appropriate immunotherapy in the preoperative period, this immunosuppression may be prevented or reversed. This could lead to fewer infectious complications and accelerated wound healing. Second, tumor bulk is minimal following surgery and immunotherapy is most likely to be effective in this situation. A third reason is the possibility that tumor cells are shed into the circulation at surgery and effective immunotherapy applied at this time can eliminate these cells.

There are two types of immunotherapy, the "active immunotherapy" and the "passive immunotherapy". In "active immunotherapy", an antigen is administered in the form of a vaccine, to a patient, so as to elicit a protective immune response. "Passive immunotherapy" involves the administration of antibodies to a patient without eliciting a concomitant immune response. When a specific antibody from one animal is injected as

an immunogen into a suitable second animal, the injected antibody will elicit an immune response. Antibody therapy is conventionally characterized as passive since the patient is not the source of the antibodies. However, the term passive is misleading because the patient can produce anti-idiotypic secondary antibodies which in turn provoke an immune response which is cross-reactive with the original antigen. Immunotherapy where the patient generates secondary antibodies is often more therapeutically effective than passive immunotherapy because the patient's own immune system continues to fight the cells bearing the particular antigen well after the initial infusion of antibody.

In an anti-idiotypic response, antibodies produced initially during an immune response or introduced into an organism will carry unique new epitopes to which the organism is not tolerant, and therefore will elicit production of secondary antibodies (termed "Ab2"), some of which are directed against the idiotype (*i.e.*, the antigen binding site) of the primary antibody (termed "Ab1"), *i.e.*, the antibody that was initially produced or introduced exogenously. These secondary antibodies or Ab2 likewise will have an idiotype, which will induce production of tertiary antibodies (termed "Ab3"), some of which will recognize the antigen binding site of Ab2, and so forth. This is known as the "network" theory. Some of the secondary antibodies will have a binding site which is an analog of the original antigen, and thus will reproduce the "internal image" of the original antigen. And, the tertiary or Ab3 antibodies that recognize this antigen binding site of the Ab2 antibody will also recognize the original antigen (Figure 4).

Therefore, anti-idiotypic antibodies have binding sites that are similar in conformation and charge to the antigen, and can elicit the same or greater response than that of the cancer antigen itself. Administration of an exogenous antibody that can elicit a strong anti-idiotypic response can thus serve as an effective vaccine, by maintaining a constant immune response.

To date, anti-idiotypic vaccines have comprised murine antibodies because the anti-idiotypic response occurs as part of the typical human anti-mouse antibody (HAMA) response. A strong anti-idiotypic cascade has been observed when Ab1 has been structurally damaged (Madiyalakan et al., 1995, *Hybridoma* 14:199-203), rendering the antibody more foreign. There has been direct administration to the subject of exogenously produced anti-idiotypic antibodies that are raised against the idiotype of an anti-tumor antibody (U.S. Patent No. 4,918,14). After administration, the subject's body will produce anti-antibodies which not only recognize these anti-idiotypic antibodies, but also recognize the original tumor epitope, thereby directing complement activation and other immune system responses to a foreign entity to attack the tumor cell that expresses the tumor epitope.

However, while anti-idiotypic vaccines are desirable targets and several have been identified, the ability to deliver antibodies that reproducibly cause the generation of such an anti-idiotypic response is not currently possible. (Foon et al., 1995, *J. Clin. Invest.* 9:334-342; Madiyalakan et al., 1995, *Hybridoma* 14:199-203). One of the reasons for the failure
5 to generate an anti-idiotypic response is that, Ab1, while exogenous, is still very similar to "self", as all antibodies have very similar structures, and anti-idiotypic responses to self molecules tend to be very limited. Thus, there is a need in the art for methods of reliably generating an anti-idiotypic response to a specific antibody.

10

2.3. CONTRACEPTIVE METHODS

A variety of contraceptive methods are currently available. Such methods include barrier methods such as condoms or diaphragms, or use of spermicidal agents such as non-oxynol-9, hormone therapies such as birth control pills or implants, and other methods such as intrauterine devices. All of these methods pose problems as convenient and effective
15 methods of preventing conception. Some methods are inconvenient or ineffective, some pose health risks, while others are costly. Accordingly, there is a need in the art for a safe, inexpensive, and convenient method of contraception.

3. SUMMARY OF THE INVENTION

20

The present invention is based upon the realization of the present inventors that an antibody in which one or more variable region cysteine residues that form one or more intrachain disulfide bonds have been replaced with amino acid residues that do not contain
25 sulfhydryl groups, such that the particular disulfide bonds do not form, elicit a much stronger anti-idiotypic response than an antibody in which the variable region disulfide bonds are intact. Additionally, the present inventors have realized that portions of antigens of proteins or reproductive cells, particularly sperm antigens, can be inserted into or used to replace portions of one or more complementarity determining regions, such that the modified antibody can be used as a vaccine to generate anti-idiotypic antibodies that recognize the particular antigen.

30

Accordingly, the present invention provides modified immunoglobulin molecules or antibodies (and functionally active fragments, derivatives and analogs thereof), and vaccine compositions containing these immunoglobulin molecules, wherein the variable region of the immunoglobulin is subject to decreased conformational constraints, such as, but not limited to, by breaking one or more intrachain or interchain disulfide bonds. Specifically,
35 the invention provides modified immunoglobulins that comprise a variable region and are identical, except for one or more amino acid substitutions in said variable region, to a

second immunoglobulin molecule, said second immunoglobulin molecule being capable of immunospecifically binding (*i.e.*, specific binding of the immunoglobulin to its antigen as determined by any method known in the art for determining antibody-antigen binding, which excludes non-specific binding but not necessarily cross-reactivity with other
 5 antigens) an antigen or having a CDR that contains a portion of an antigen, said one or more amino acid substitutions being the substitution of one or more amino acid residues that do not have a sulfhydryl group at one or more positions corresponding to one or more cysteine residues that form a disulfide bond in said second immunoglobulin molecule. In preferred
 10 embodiments, the second immunoglobulin molecule contains a CDR that contains a portion of an antigen of a cell or protein involved in reproductive function, preferably sperm antigens, more preferably the sperm antigens SP-10, LDH-C₄, or MSA-63.

The invention further provides methods of eliciting an anti-idiotypic response in a subject by administering the modified immunoglobulins of the invention. In particular, the modified immunoglobulins of the invention can be used as contraceptives, either in males
 15 or, preferably in females, specifically by administering an immunoglobulin molecule of the invention, which immunoglobulin molecule was derived (*i.e.*, by modification according to the invention to replace one or more variable region cysteine residues that form an intrachain disulfide bond with an amino acid residue that does not contain a sulfhydryl
 20 group) from an immunoglobulin molecule that contains a CDR that contains a portion of an antigen of a protein or cell associated with reproductive function, preferably a sperm antigen.

The invention also provides methods of producing the modified immunoglobulin molecules of the invention and vaccine compositions containing the modified immunoglobulin molecules of the invention.

25

4. DESCRIPTION OF FIGURES

Figure 1. A schematic diagram showing the structure of the light and heavy chain of an immunoglobulin molecule, each chain consisting of a variable region positioned at the amino terminal region (H₂N-) and a constant region positioned at a carboxyl terminal region
 30 (-COOH).

Figure 2. A schematic diagram of an IgG showing the four framework regions (FR1, FR2, FR3 and FR4) and three complementarity determining regions (CDR1, CDR2 and CDR3) in the variable regions of the light and heavy chains (labeled as V_L and V_H, respectively). The constant region domains are indicated as C_L for the light chain constant
 35 domain and CH₁, CH₂ and CH₃ for the three domains of the heavy chain constant region. Fab indicates the portion of the antibody fragment which includes the variable region

domains of both light and heavy chains and the C_L and CH_1 domains. F_c indicates the constant region fragment containing the CH_2 and CH_3 domains.

Figure 3. A schematic diagram of an antibody structure as shown in Figure 2, but drawn to emphasize that each domain (the loop structures labeled as V_L , V_H , C_L , CH_1 , CH_2 , and CH_3 , respectively) is structurally defined by a disulfide bond (indicated with darkest lines) that maintains the three-dimensional structure (Roitt et al., *Immunology*, Second Edition, London: Gower Medical Publishing, 1989, p 5.3).

Figure 4. A schematic diagram showing the development of internal image bearing anti-idiotypic antibodies (Ab2) and anti-anti-idiotypic antibodies (Ab3) from idiotype antibodies (Ab1) directed against an antigen of a tumor cell in an antiidiotypic cascade.

Figure 5. Modification of the variable region of an immunoglobulin by replacing cysteine residues in the variable regions with alanine residues to remove an intrachain disulfide bond. CH_1 , CH_2 and CH_3 are constant regions. V_H is the heavy chain variable region and V_L is the light chain variable region.

Figures 6A-C. (A). The structure of the expression vector pMRRO10.1, which contains a human kappa light chain constant region sequence. (B). The structure of the expression vector pGamma1 that contains a sequence encoding a human IgG1 constant region (CH_1 , CH_2 , CH_3) heavy chain and hinge region sequences. (C) The structure of the expression vector pNEPuDGV which contains a sequence encoding the kappa constant domain of the light chain and the constant domain and hinge region of the heavy chain. For all three vectors see Bebbington et al., 1991, *Methods in Enzymology* 2:136-145.

Figures 7A and B. (A) The amino acid sequence and corresponding nucleotide sequence for the consensus light chain variable region ConVL1. (B) The amino acid and corresponding nucleotide sequences for the consensus heavy chain variable region ConVH1.

Figure 8. A schematic diagram of the general steps that were followed for the assembly of an engineered gene encoding the synthetic modified antibody specific to human colon cancer antigen.

Figure 9. Dot blot showing the result of an assay for the competition of binding of the antibody derived from mAB31.1, but not having the cysteine to alanine changes with the same antibody which is biotin labeled to an antigen preparation derived from LS-174 T-cells. The concentration of the unlabeled antibody is indicated as nM unlabeled antibody. The "blk" lane has no antigen.

Figures 10A-D. (A) Results of competition binding assay of the biotin-labeled anti-colon carcinoma cell antibody to LS-174T cells in the presence of antisera from mice vaccinated with vehicle alone, control antibody that binds the colon carcinoma cell antibody

but has not been modified, and peptides CDR1, CDR2, CDR3, CDR4, CDR5, and CDR6, having the CDR sequences containing the bradykinin receptor binding site expressed as percent of control binding to LS-174T cells. (B). Results of competition binding assays of the biotin-labeled anti-colon carcinoma cell antibody to LS-174T cells in the presence of antisera from mice vaccinated with vehicle alone, control antibody that binds the colon carcinoma cell antibody, but has not been modified, 2CAVHCOL1, and 2CAVLCOL1. (C) Diagram showing the binding of a biotin-labeled (indicated by the "b") antibody (inverted Y) to antigen (solid triangles). (D) Diagram showing the inhibition of binding of the biotin-labeled (indicated by the "b") antibody (inverted Y) by anti-idiotypic antibodies (solid arrows) to antigen (solid triangles).

Sub B2 Figure 11. Nucleotide sequences of the oligonucleotides used to construct the MSA1 and MSALVAC-1 variable regions.

Sub B3 Figures 12A-C. (A) Nucleotide sequence for the MSA-63 epitope. (B) Amino acid sequence of the MSA-63 epitope encoded by the nucleotide sequence of Figure 12A. (C) MSA-63 oligonucleotides used to construct a modified variable region. Each oligo overlaps for five codons and transitions the entire sequence of Figure 12A.

Sub B4 Figures 13 A-C. (A) Nucleotide sequence for the SP-10 epitope. (B) Amino acid sequence of the SP-10 epitope encoded by the nucleotide sequence of Figure 13A. (C) Oligonucleotides of Sp-10 used to construct a modified variable region. SP3a and SP4a are modified to change the codons encoding certain cysteine residues to codons encoding alanine residues.

Sub B5 Figure 14. Oligonucleotides of LDH-C4 epitope sequence for construction of a modified variable region gene containing a LDH-C₄.

Sub B6 Figure 15. Nucleotide and amino acid sequence of the consensus contraceptive light chain variable region.

Sub B7 Figure 16 A-B. (A) Sequences of oligos used in the construction of 2CAVHCOL1. (B) Sequences of oligos used in the construction of 2CAVLCOL1.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides modified immunoglobulins (particularly antibodies and functionally active fragments, derivatives, and analogs thereof) that can be used as contraceptive vaccines. Specifically, these antibodies have one or more complementarity determining regions (CDRs) that contain a portion of an antigen of a cell or protein involved in reproductive function, preferably a sperm antigen. In addition, these antibodies have been engineered to elicit a stronger immune response, particularly a stronger anti-idiotypic response, than the corresponding unmodified immunoglobulins. In particular, the

modified immunoglobulins of the invention are immunoglobulins that are modified to decrease the conformational constraints on one variable region of the immunoglobulin molecule, preferably, such that at least one of the cysteines that participates in forming an intrachain disulfide bond in the variable region of the immunoglobulin has been replaced
5 with an amino acid residue that does not have a sulfhydryl group and, therefore, does not form a disulfide bond, thereby decreasing the conformational constraints of at least one of the variable regions of the immunoglobulin (Figure 5).

The invention also provides vaccine compositions containing the modified immunoglobulin molecules of the invention. Additionally, the invention provides methods
10 of generating an anti-idiotypic response in a subject by administration of the modified immunoglobulin molecules of the invention.

In specific embodiments, the invention provides methods of contraception by administration of a modified immunoglobulin molecule of the invention which, in its unmodified state, is capable of immunospecifically binding an antigen of a protein or cell
15 associated with reproductive function, such as a sperm antigen. Administration of the modified immunoglobulin elicits an anti-idiotypic reaction in the subject, leading to the production, by the subject, of antibodies specific for the particular antigen.

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections which follow.

20

5.1. MODIFIED ANTIBODIES

The modified immunoglobulins, particularly antibodies, of the invention are immunoglobulins that, at least in the unmodified state, can immunospecifically bind an antigen of a cell or protein associated with reproductive function, and have been modified to
25 enhance their ability to elicit an anti-idiotypic response. Such immunoglobulins are modified to reduce the conformational constraints on a variable region of the immunoglobulin, *e.g.*, by removing or reducing intrachain or interchain disulfide bonds. Specifically, the invention provides a first immunoglobulin molecule that comprises a variable region and that is identical, except for one or more amino acid substitutions in the
30 variable region, to a second immunoglobulin molecule, the second immunoglobulin molecule being capable of immunospecifically binding an antigen, the amino acid substitutions being the substitution of one or more amino acid residues that do not have a sulfhydryl group at one or more positions corresponding to one or more cysteine residues that form a disulfide bond in said second immunoglobulin molecule. (See, co-pending
35 United States Patent Application Serial No., entitled "Modified Antibodies With Enhanced Ability To Elicit An Anti-Idiotypic Response", filed November 13, 1998 (attorney docket

no. 6750-015), which is incorporated by reference herein in its entirety. The invention also provides nucleic acids containing a nucleotide sequence encoding a modified immunoglobulin of the invention.

Identifying the cysteine residues that form a disulfide bond in a variable region of a particular antibody can be accomplished by any method known in the art. For example, but not by way of limitation, it is well known in the art that the cysteine residues that form intrachain disulfide bonds are highly conserved among antibody classes and across species. Thus, the cysteine residues that participate in disulfide bond formation can be identified by sequence comparison with other antibody molecules in which it is known which residues form a disulfide bond.

Table 1 provides a list of the positions of disulfide bond forming cysteine residues for a number of antibody molecules.

Table 1 (derived from Kabat et al, 1991, sequences of Proteins of Immunological Interest, 5th Ed., U.S. Department of Health and Human Services, Bethesda, Maryland).

15

Species	Variable domain	Subgroup	Disulfide bond-forming
			cysteines (positions)
Human	kappa light	I	23,88
20 Human	kappa light	II	23,88
Human	kappa light	III	23,88
Human	kappa light	IV	23,88
Human	lambda light	I	23,88
Human	lambda light	II	23,88
Human	lambda light	III	23,88
Human	lambda light	IV	23,88
25 Human	lambda light	V	23,88
Human	lambda light	VI	23,88
Mouse	kappa light	I	23,88
Mouse	kappa light	II	23,88
Mouse	kappa light	III	23,88
Mouse	kappa light	IV	23,88
30 Mouse	kappa light	V	23,88
Mouse	kappa light	VI	23,88
Mouse	kappa light	VII	23,88
Mouse	kappa light	Miscellaneous	23,88
Mouse	lambda light		23,88
Chimpanzee	lambda light		23,88
Rat	kappa light		23,88
35 Rat	lambda light		23,88
Rabbit	kappa light		23,88
Rabbit	lambda light		23,88

		Disulfide bond-forming	
		cysteines	
Species	Variable domain	Subgroup	(positions)
	Dog	kappa light	23,88
	Pig	kappa light	23 (88)
5	Pig	lambda light	23,88
	Guinea pig	lambda light	23 (88)
	Sheep	lambda light	23,88
	Chicken	lambda light	23,88
	Turkey	lambda light	23 (88)
	Ratfish	lambda light	23 (88)
10	Shark	kappa light	23,88
	Human	heavy I	22,92
	Human	heavy II	22,92
	Human	heavy III	22,92
	Mouse	heavy I (A)	22,92
	Mouse	heavy I (B)	22,92
	Mouse	heavy II (A)	22,92
15	Mouse	heavy II (B)	22,92
	Mouse	heavy II (C)	22,92
	Mouse	heavy III (A)	22,92
	Mouse	heavy III(B)	22,92
	Mouse	heavy III (C)	22,92
	Mouse	heavy III (D)	22,92
	Mouse	heavy V (A)	22,92
20	Mouse	heavy V (B)	22,92
	Mouse	Miscellaneous	22,92
	Rat	heavy	22,92
	Rabbit	heavy	22,92
	Guinea pig	heavy	22,92
	Cat	heavy	22 (92)
25	Dog	heavy	22,92
	Pig	heavy	22 (92)
	Mink	heavy	22 (92)
	Sea lion	heavy	22 (92)
	Seal	heavy	22 (92)
	Chicken	heavy	22,92
	Duck	heavy	22 (92)
30	Goose	heavy	22 (92)
	Pigeon	heavy	22 (92)
	Turkey	heavy	22 (92)
	Caiman	heavy	22, 92
	Xenopus frog	heavy	22,92
	Elops	heavy	22,92
	Goldfish	heavy	22,92
35	Ratfish	heavy	22 (92)
	Shark	heavy	22,92

Position numbers enclosed by () indicate that the protein was not sequenced to that position, but the residue is inferred by comparison to known sequences.

Notably, for all of the antibody molecules listed in Table 1, the cysteine residues that form the intrachain disulfide bonds are the residues at positions 23 and 88 of the light chain variable domain and the residues at positions 22 and 92 of the heavy chain variable domain. The position numbers refer to the residue corresponding to that residue in the consensus sequences as defined in Kabat, (1991, Sequences of Proteins of Immunological Interest, 5th Ed., U.S. Department of Health and Human Services, Bethesda, Maryland) or as indicated in the heavy and light chain variable region sequences depicted in Figures 7A and B, respectively ("corresponding" means as determined by aligning the particular antibody sequence with the consensus sequence or the heavy or light chain variable region sequence depicted in Figure 7A or B).

Accordingly, in one embodiment of the invention, the modified immunoglobulin molecule is an antibody in which the residues at positions 23 and/or 88 of the light chain are substituted with an amino acid residue that does not contain a sulfhydryl group and/or the residues at positions 22 and/or 92 of the heavy chain are substituted with an amino acid residue that does not contain a sulfhydryl group.

In the modified immunoglobulin of the invention, the amino acid residue that substitutes for the disulfide bond forming cysteine residue is any amino acid residue that does not contain a sulfhydryl group, *e.g.*, alanine, arginine, asparagine, aspartate (or aspartic acid), glutamine, glutamate (or glutamic acid), glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine or valine. In a preferred embodiment, the cysteine residue is replaced with a glycine, serine, threonine, tyrosine, asparagine, or glutamine residue, most preferably, with an alanine residue.

Additionally, the disulfide bond forming cysteine residue may be replaced by a nonclassical amino acid or chemical amino acid analog that does not contain a sulfhydryl group (for example, but not by way of limitation, using routine protein synthesis methods). Non-classical amino acids include, but are not limited, to the D-isomers of the common amino acids, α -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-aminobutyric acid, γ -Abu, ϵ -Ahx, -amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β -alanine, fluoro-amino acids, designer amino acids such as β -methyl amino acids, C α -methyl amino acids, N α -methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary). In an alternative embodiment, the disulfide bond forming residue is deleted.

In specific embodiments, the substitution of the disulfide bond forming residue is in the heavy chain variable region or is in the light chain variable region or is in both the heavy chain and light chain variable regions. In other specific embodiments, one of the residues that forms a particular disulfide bond is replaced (or deleted) or, alternatively, both residues
 5 that form a particular disulfide bond may be replaced (or deleted).

In other embodiments, the invention provides immunoglobulin molecules that have one or more amino acid substitutions relative to the second immunoglobulin molecule of a disulfide bond forming residue in the variable region with an amino acid residue that does not contain a sulfhydryl group and that additionally have one or more other amino acid
 10 substitutions (*i.e.*, that are not a replacement of a disulfide bond forming residue with a residue that does not contain a sulfhydryl group).

In particular, the invention provides a first immunoglobulin molecule comprising a variable region and which is identical, except for one or more amino acid substitutions in said variable region, to a second immunoglobulin molecule, said second immunoglobulin
 15 molecule being capable of immunospecifically binding an antigen of a cell or protein associated with reproductive function or that has at least one CDR that contains a portion of an antigen of a cell or protein associated with reproductive function, in which at least one of said one or more amino acid substitutions are the substitution of an amino acid residue that does not have a sulfhydryl group at one or more positions corresponding to one or more
 20 cysteine residues that form a disulfide bond in said second immunoglobulin molecule.

In a preferred embodiment, the amino acid substitutions that are not the substitution of a disulfide bond forming cysteine residue with a residue that does not have a sulfhydryl group, are not stabilizing changes. Stabilizing changes are defined as those amino acid changes that increase the stability of the antibody molecule. Such stabilizing amino acid
 25 changes are those changes that substitute an amino acid that is not common at that particular position in the particular antibody molecule (*e.g.*, as defined by the consensus sequences for a number of antibody molecules provided in Kabat et al., 1991, Sequences of Proteins of Immunological Interest, 5th Ed., U.S. Department of Health and Human Services, Bethesda, Maryland) with a residue that is common at that particular position, *e.g.*, is the amino acid
 30 at that position in the consensus sequence for that antibody molecule (see PCT Publication WO 96/02574, dated February 1, 1996 by Steipe et al.).

Such other amino acid substitutions can be any amino acid substitution that does not alter the ability of the modified immunoglobulin to elicit the formation of anti-anti-idiotypic antibodies, *e.g.*, as determined, for example, as described in Section 5.5, *infra*. For
 35 example, such other amino acid substitutions include substitutions of functionally equivalent amino acid residues. For example, one or more amino acid residues can be

substituted by another amino acid of a similar polarity which acts as a functional equivalent. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

The modified immunoglobulin is derived from an antibody that has one or more CDRs containing a portion of an antigen of a cell or protein associated with reproductive function. In specific embodiments, the antigen is a sperm antigen, preferably SP-10. Other antigens include lactate dehydrogenase LDH-C4, SP-17, PH-20, FA-1, FA-2, PH-30, RSA, HAS-63, MSA-63, or zona pellucida proteins ZP1, ZP2, and ZP3 (see, e.g., Freemerman et al., 1993, *Molecular Reproduction and Development* 34:140-148; Herr et al., 1990, *Biol. Reproduction* 42:181-193; O'Hern et al., 1995, *Biol. Reproduction* 52:331-339; Anderson et al., 1986, *J. Reprod. Immunol.* 10:231-257; Wright et al., 1990, *Biology of Reproduction* 42:693-701; Lea et al., 1997, *Fertility and Sterility* 67:355-361; O'Hern et al., Elsevier Science Ltd. 16:1761-1766; Kerr, 1995, *Reprod. Fertil. Dev.* 7:825-830; Kaul et al., 1996, *Reprod. Fertil. Dev.* 50:127-134; Liu et al., 1990, *Molecular Reproduction and Development* 25:302-308; Bambra, 1992, *Scand. J. Immunol.* 11:118-122) or another antigen of a cell or protein associated with reproductive function, for example but not limited to gonadotropin-releasing hormone, any gonadotropin, prostaglandin F2 alpha, oxytocin, and gonadotropin receptors.

The immunoglobulin molecules of the invention can be of any type, class, or subclass of immunoglobulin molecules. In a preferred embodiment, the immunoglobulin molecule is an antibody molecule, more preferably of a type selected from the group consisting of IgG, IgE, IgM, IgD and IgA, most preferably is an IgG molecule. Alternatively, the immunoglobulin molecule is a T cell receptor, a B cell receptor, a cell-surface adhesion molecule such as the co-receptors CD4, CD8, or CD19, or an invariant domain of an MHC molecule.

The modified immunoglobulin can be derived from any naturally occurring antibody, preferably a monoclonal antibody, or can be derived from a synthetic or engineered antibody. Specifically, the modified immunoglobulin molecules are derived from an antibody in which a portion of an antigen of a cell or protein associated with reproductive function is inserted into or replaces all or a portion of one of the CDRs in the variable region, for example as described in co-pending United States Patent application

Serial No., entitled "Immunoglobulin Molecules Having A Synthetic Variable Region And Modified Specificity", by Burch, filed November 13, 1998 (attorney docket no. 6750-016), which is incorporated by reference herein in its entirety.

In particular, the synthetic antibodies are antibodies that in which at least one of the
 5 CDRs of the antibody contains an antigen of a cell or protein associated with reproductive function. In one aspect of the invention, the amino acid sequence of the antigen is not found naturally within the CDR. One or more CDRs may also contain a binding site for a cell or protein involved in reproductive function.

The amino acid sequence of the binding site may be identified by any method
 10 known in the art. For example, in some instances, the sequence of a member of a binding pair has already been determined to be directly involved in binding the other member of the binding pair. In this case, such a sequence can be used to construct the CDR of a synthetic antibody that specifically recognizes the other member of the binding pair. If the amino acid sequence for the binding site in the one member of the binding pair for the other
 15 member of the binding pair is not known, it can be determined by any method known in the art, for example, but not limited to, molecular modeling methods or empirical methods, *e.g.*, by assaying portions (*e.g.*, peptides) of the member for binding to the other member, or by making mutations in the member and determining which mutations prevent binding.

The binding pair can be any two molecules, including proteins, nucleic acids,
 20 carbohydrates, or lipids, that interact with each other, although preferably the binding partner from which the binding site is derived is a protein molecule. In preferred embodiments, the modified immunoglobulin contains a binding sequence for a cancer antigen, an infectious disease antigen, a cellular receptor for a pathogen, or a receptor or ligand that participates in a receptor-ligand binding pair.

25 In specific embodiments, the binding pair is a protein-protein interaction pair which is either homotypic interaction (*i.e.*, is the interaction between two of the same proteins) or a heterotypic interaction (*i.e.*, is the interaction between two different proteins).

The synthetic antibody may be built upon (*i.e.*, the binding site sequences inserted into the CDR of) the sequence of a naturally occurring or previously existing antibody or
 30 may be synthesized from known antibody consensus sequences, such as the consensus sequences for the light and heavy chain variable regions in Figures 7A and B, or any other antibody consensus or germline (*i.e.*, unrecombined genomic sequences) sequences (*e.g.*, those antibody consensus and germline sequences described in Kabat et al., 1991, Sequences of Proteins of Immunological Interest, 5th edition, NIH Publication No. 91-3242,
 35 pp 2147-2172).

Each antibody molecule has six CDR sequences, three on the light chain and three on the heavy chain, and five of these CDRs are germline CDRs (*i.e.*, are directly derived from the germline genomic sequence of the animal, without any recombination) and one of the CDRs is a non-germline CDR (*i.e.*, differs in sequence from the germline genomic sequence of the animal and is generated by recombination of the germline sequences).
 5 Whether a CDR is a germline or non-germline sequence can be determined by sequencing the CDR and then comparing the sequence with known germline sequences, *e.g.*, as listed in Kabat et al. (1991, Sequences of Proteins of Immunological Interest, 5th edition, NIH Publication No. 91-3242, pp 2147-2172). Significant variation from the known germline
 10 sequences indicates that the CDR is a non-germline CDR. Accordingly, the CDR that contains the amino acid sequence of the binding site or antigen is a germline CDR or, alternatively, is a non-germline CDR.

The binding site or antigen sequence can be inserted into any of the CDRs of the antibody, and it is within the skill in the art to insert the binding site into different CDRs of
 15 the antibody and then screen the resulting modified antibodies for the ability to bind to the particular member of the binding pair, *e.g.* as discussed in Section, *infra*, or to elicit an immune response against the antigenic site, *e.g.*, as described in Section, *infra*. Thus, one can determine which CDR optimally contains the binding site or antigen. In specific embodiments, a CDR of either the heavy or light chain variable region is modified to
 20 contain the amino acid sequence of the binding site or antigen. In another specific embodiment, the modified antibody contains a variable domain in which the first, second or third CDR of the heavy variable region or the first, second or third CDR of the light chain variable region contains the amino acid sequence of the binding site or antigen. In another embodiment of the invention, more than one CDR contains the amino acid sequence of the
 25 binding site or antigen or more than one CDR each contains a different binding site for the same molecule or contains a different binding site for a different molecule. In particular, embodiments, two, three, four, five or six CDRs have been engineered to contain a binding site for the first member of the binding pair. In a preferred embodiment, one of the CDRs contains a portion of one sperm antigen and another CDR contains a portion of a second
 30 sperm antigen, more particularly, where one sperm antigen is SP-10 and the other sperm antigen is MSA-63 or LHD-C₄.

In specific embodiments of the invention, the binding site or antigen amino acid sequence is either inserted into the CDR without replacing any of the amino acid sequence of the CDR itself or, alternatively, the binding site or antigen amino acid sequence replaces
 35 all or a portion of the amino acid sequence of the CDR. In specific embodiments, the

binding site amino acid sequence replaces 1, 2, 5, 8, 10, 15, or 20 amino acids of the CDR sequence.

The amino acid sequence of the binding site or antigen present in the CDR can be the minimal binding site necessary for the binding of the member of the binding pair or for eliciting an immune response against the antigen(which can be determined empirically by any method known in the art); alternatively, the sequence can be greater than the minimal binding site or antigen sequence necessary for the binding of the member of the binding pair or eliciting of an immune response against the antigen. In particular embodiments, the binding site or antigen amino acid sequence is at least 4 amino acids in length, or is at least 6, 8, 10, 15, or 20 amino acids in length. In other embodiments the binding site amino acid sequence is no more than 10, 15, 20, or 25 amino acids in length, or is 5-10, 5-15, 5-20, 10-15, 10-20 or 10-25 amino acids in length.

In addition, the total length of the CDR (*i.e.*, the combined length of the binding site sequence and the rest of the CDR sequence) should be of an appropriate number of amino acids to allow binding of the antibody to the antigen. CDRs have been observed to have a range of numbers of amino acid residues, and the observed size ranges for the CDRs (as denoted by the abbreviations indicated in figure 2) are provided in Table 1.

Table 1

	CDR	Number of residues
20	L1	10-17
	L2	7
	L3	7-11
	H1	5-7
25	H2	9-12
	H3	2-25

(compiled from data in Kabat and Wu, 1971, *Ann. NY Acad. Sci.* 190:382-93)

While many CDR H3 regions are of 5-9 residue in length, certain CDR H3 regions have been observed that are much longer. In particular, a number of antiviral antibodies have heavy chain CDR H3 regions of 17-24 residues in length.

Accordingly, in specific embodiments of the invention, the CDR containing the binding site or antigen portion is within the size range provided for that particular CDR in Table 1, *i.e.*, if it is the first CDR of the light chain, L1, the CDR is 10 to 17 amino acid residues; if it is the second CDR of the light chain, L2, the CDR is 7 amino acid residues; if it is the third CDR of the light chain, L3, the CDR is 7 to 11 amino acid residues; if it is the

first CDR of the heavy chain, H1, the CDR is 5 to 7 amino acid residues; if it is the second CDR of the heavy chain, H2, the CDR is 9 to 12 amino acid residues; and if it is the third CDR of the heavy chain, H3, the CDR is 2 to 25 amino acid residues. In other specific embodiments, the CDR containing the binding site is 5-10, 5-15, 5-20, 11-15, 11-20, 11-25, or 16-25 amino acids in length. In other embodiments, the CDR containing the binding site is at least 5, 10, 15, or 20 amino acids or is no more than 10, 15, 20, 25, or 30 amino acids in length.

After constructing antibodies containing modified CDRs, the modified antibodies can be further altered and screened to select an antibody having higher affinity or specificity. Antibodies having higher affinity or specificity for the target antigen may be generated and selected by any method known in the art. For example, but not by way of limitation, the nucleic acid encoding the synthetic modified antibody can be mutagenized, either randomly, *i.e.*, by chemical or site-directed mutagenesis, or by making particular mutations at specific positions in the nucleic acid encoding the modified antibody, and then screening the antibodies exposed from the mutated nucleic acid molecules for binding affinity for the target antigen. Screening can be accomplished by testing the expressed antibody molecules individually or by screening a library of the mutated sequences, *e.g.*, by phage display techniques (see, *e.g.*, U. S. Patent Nos. 5,223,409; 5,403,484; and 5,571,698; all by Ladner et al; PCT Publication WO 92/01047 by McCafferty et al. or any other phage display technique known in the art).

In specific embodiments, the invention provides a functionally active fragment, derivative or analog of the modified immunoglobulin molecules of the invention. Functionally active means that the fragment, derivative or analog is able to elicit anti-idiotype antibodies (*i.e.*, tertiary antibodies or Ab3 antibodies) that recognize the same antigen that the antibody from which the fragment, derivative or analog is derived recognized (*e.g.*, as determined by the methods described in Section 5.4, *infra*). Specifically, in a preferred embodiment, the antigenicity of the idiotype of the immunoglobulin molecule may be enhanced by deletion of framework and CDR sequences that are N-terminal to the particular CDR sequence that specifically recognizes the antigen. To determine which CDR sequences bind the antigen, synthetic peptides containing the CDR sequences can be used in binding assays with the antigen by any binding assay method known in the art. Accordingly, in a preferred embodiment, the invention includes modified immunoglobulin molecules that have one disulfide bond forming cysteine residue in a variable region domain replaced with an amino acid residue that does not contain a sulfhydryl group and in which a portion of that variable domain has been deleted N-terminal to the CDR sequence that recognizes the antigen.

Other embodiments of the invention include fragments of the modified antibodies of the invention such as, but not limited to, $F(ab')_2$ fragments, which contain the variable region, the light chain constant region and the CH1 domain of the heavy chain can be produced by pepsin digestion of the antibody molecule, and Fab fragments, which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. The invention also provides heavy chain and light chain dimers of the modified antibodies of the invention, or any minimal fragment thereof such as Fvs or single chain antibodies (SCAs) (e.g., as described in U.S. Patent 4,946,778; Bird, 1988, *Science* 242:423-42; Huston et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:5879-5883; and Ward et al., 1989, *Nature* 334:544-54), or any other molecule with the same specificity as the modified antibody of the invention.

Techniques have been developed for the production of "chimeric antibodies" (Morrison et al., 1984, *Proc. Natl. Acad. Sci.* 81:851-855; Neuberger et al., 1984, *Nature* 312:604-608; Takeda et al., 1985, *Nature* 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine monoclonal antibody and a constant domain from a human immunoglobulin, e.g., humanized antibodies.

In a preferred embodiment, the modified immunoglobulin of the invention is a humanized antibody, more preferably an antibody having a variable domain in which the framework regions are from a human antibody and the CDRs are from an antibody of a non-human animal, preferably a mouse (see, International Patent Application No. PCT/GB8500392 by Neuberger et al. and Celltech Limited).

CDR grafting is another method of humanizing antibodies. It involves reshaping murine antibodies in order to transfer full antigen specificity and binding affinity to a human framework (Winter et al. U.S. Patent No. 5,225,539). CDR-grafted antibodies have been successfully constructed against various antigens, for example, antibodies against IL-2 receptor as described in Queen et al., 1989 (*Proc. Natl. Acad. Sci. USA* 86:10029); antibodies against cell surface receptors-CAMPATH as described in Riechmann et al. (1988, *Nature*, 332:323); antibodies against hepatitis B in Cole et al. (1991, *Proc. Natl. Acad. Sci. USA* 88:2869); as well as against viral antigens-respiratory syncytial virus in Tempest et al. (1991, *Bio-Technology* 9:267). CDR-grafted antibodies are generated in which the CDRs of the murine monoclonal antibody are grafted into a human antibody. Following grafting, most antibodies benefit from additional amino acid changes in the framework region to maintain affinity, presumably because framework residues are necessary to maintain CDR conformation, and some framework residues have been

demonstrated to be part of the antigen binding site. However, in order to preserve the framework region so as not to introduce any antigenic site, the sequence is compared with established germline sequences followed by computer modeling.

In other embodiments, the invention provides fusion proteins of the modified immunoglobulins of the invention (or functionally active fragments thereof), for example in which the modified immunoglobulin is fused via a covalent bond (*e.g.*, a peptide bond), at either the N-terminus or the C-terminus to an amino acid sequence of another protein (or portion thereof, preferably an at least 10, 20 or 50 amino acid portion of the protein) that is not the modified immunoglobulin. Preferably the modified immunoglobulin, or fragment thereof, is covalently linked to the other protein at the N-terminus of the constant domain. In preferred embodiments, the invention provides fusion proteins in which the modified immunoglobulin is covalently linked to IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, γ -interferon, MHC derived peptide, G-CSF, a porin, TNF, NK cell antigens, or cellular endocytosis receptor.

The modified immunoglobulins of the invention include analogs and derivatives that are either modified, *i.e.*, by the covalent attachment of any type of molecule as long as such covalent attachment does not prevent the modified immunoglobulin from generating an anti-idiotypic response (*e.g.*, as determined by any of the methods described in Section 5.5, *infra*). For example, but not by way of limitation, the derivatives and analogs of the modified immunoglobulins include those that have been further modified, *e.g.*, by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the analog or derivative may contain one or more non-classical amino acids, *e.g.*, as listed above in this Section.

Methods of producing the modified immunoglobulins, and fragments, analogs, and derivatives thereof, are described in Section 5.4, *infra*.

30

5.2. CONTRACEPTIVE METHODS

The present invention provides methods of contraception by eliciting production of anti-idiotypic antibodies and anti-anti-idiotypic antibodies in a subject by the administration of a therapeutic (termed herein "Therapeutic"). Such Therapeutics include the modified immunoglobulins of the invention, and functionally active fragments, analogs, and derivatives thereof (*e.g.*, as described in Section 5.1, *supra*), and nucleic acids encoding the

35

modified antibodies of the invention, and functionally active fragments and derivatives thereof (*e.g.*, as described in Section 5.1, *supra*).

Generally, administration of products of a species origin or species reactivity that is the same species as that of the subject is preferred. Thus, in a preferred embodiment, the methods of the invention use a modified antibody that is derived from a human antibody; in other embodiments, the methods of the invention use a modified antibody that is derived from a chimeric or humanized antibody.

Specifically, vaccine compositions (*e.g.*, as described in Section 5.3, *infra*) containing the modified antibodies of the invention are administered to the subject to elicit the production of an antibody (*i.e.*, the anti-idiotypic antibody or Ab2) that specifically recognizes the idiotype of the modified antibody, the Ab2, in turn, elicits the production anti-anti-idiotypic antibodies (Ab3) that specifically recognize the idiotype of Ab2, such that these Ab3 antibodies have the same or similar binding specificity as the modified antibody.

The invention provides methods of administering the modified antibodies of the invention to elicit an anti-idiotypic response, *i.e.*, to generate Ab2 and Ab3 type antibodies. Alternatively, the invention provides methods of administering the modified antibodies of the invention to one subject to generate Ab2 antibodies, isolating the Ab2 antibodies, and then administering the Ab2 antibodies to a second subject to generate Ab3 type antibodies in that second subject.

Accordingly, the invention provides a method of generating an anti-idiotypic response in a subject comprising administering an amount of first immunoglobulin molecule (or functionally active fragment, analog, or derivative thereof) sufficient to induce an anti-idiotypic response, said first immunoglobulin comprising a variable region and being identical, except for one or more amino acid substitutions in said variable region, to a second immunoglobulin molecule, said second immunoglobulin molecule being capable of immunospecifically binding an antigen, said one or more amino acid substitutions being the substitution of an amino acid residue that does not have a sulfhydryl group at one or more positions corresponding to one or more cysteine residues that form a disulfide bond in said second immunoglobulin molecule. In another embodiment, the method further provides isolating the anti-idiotypic antibody that recognizes the idiotype of said second immunoglobulin molecule, and administering to a second subject the anti-idiotypic antibody.

Modified immunoglobulins capable of inhibiting the gamete interaction *i.e.*, of eggs and sperm are preferably employed. The key to this method of contraception is to either immunologically regulate molecules involved in reproduction or to inhibit fertilization. Such contraceptive vaccines target reproductive hormone or receptor-specific antigens or gamete-specific antigens. The goal is to elicit an immune response which targets

reproductive hormones or receptors or native gamete molecules. In preferred embodiments, the vaccine targets sperm by eliciting production of antibodies that recognize sperm antigens.

5 Fertility can be suppressed by immunization against a reproductive hormone or receptor such as gonadotropin-releasing hormone, gonadotropins, prostaglandin F2 alpha, oxytocin and gonadotropin receptors.

Fertility can also be suppressed by immunization against gamete or embryonic antigens. Fertilization is mediated through specific molecules of the sperm and egg. In mammals, the sperm and egg interact at an egg-specific extracellular matrix, the zona
 10 pellucida (ZP), and the sperm plasma membrane (Gupta et al., 1997, Hum. Reprod. Update, 3(4):311-324). The zona pellucida comprises three glycoproteins ZP1, ZP2 and ZP3 (Kaul et al., 1997, Mol. Reprod. Dev. 47(2):140-147) which are target antigens for designing immunocontraceptives. Some of the sperm plasma membrane proteins which are useful as
 15 antigens for immunocontraception are PH-20 (Primakoff et al., 1997, Biol. Reprod., 56(5):1142-1146) and PH-30 (Kerr, Reprod. Fertil. Dev., 1995, 7(4):825-830). Other sperm proteins are SP-10 (Kurth et al., 1997, Biol. Reprod., 57(5):981-989) and SP-17 (Adoyo et al., 1997, Mol. Reprod. Dev., 47(1):66-71). Other gamete proteins include lactate dehydrogenase-C4 (LDH-C4) (Bradley et al., Reprod. Fertil. Dev., 9(1):111-116) and fertilization antigen-1 (FA-1) (Zhu and Naz, Proc. Natl. Acad. Sci. USA., 94(9):4704-
 20 4709).

In particular, the contraceptive methods of the invention involve administration of modified immunoglobulin molecules (or functionally active fragments, derivatives or an analog thereof, or nucleic acids encoding the same) derived from an immunoglobulin molecule that specifically recognizes a molecule or cell involved in reproductive function.
 25 In a specific embodiment, the contraceptive methods of the invention involve the administration of a modified immunoglobulin molecule that is derived from an antibody that is capable of immunospecifically binding to gonadotropin-releasing hormone, any gonadotropin, prostaglandin F2 alpha, oxytocin, gonadotropin receptors, gamete or embryonic antigens, sperm antigens, preferably SP-10. Other antigens include, but are not
 30 limited to, lactate dehydrogenase LDH-C4, SP-17, PH-20, FA-1, FA-2, PH-30, RSA, HAS-63, MSA-63, or zona pellucida proteins ZP1, ZP2, and ZP3 (see, e.g., Freemerman et al., 1993, Molecular Reproduction and Development 34:140-148; Herr et al., 1990, Biol. Reproduction 42:181-193; O'Hern et al., 1995, Biol. Reproduction 52:331-339; Anderson et al., 1986, J. Reprod. Immunol. 10:231-257; Wright et al., 1990, Biology of Reproduction 42:693-701; Lea et al., 1997, Fertility and Sterility 67:355-361; O'Hern et al., Elsevier
 35 Science Ltd. 16:1761-1766; Kerr, 1995, Reprod. Fertil. Dev. 7:825-830; Kaul et al., 1996,

Reprod. Fertil Dev. 50:127-134; Liu et al., 1990, Molecular Reproduction and Development 25:302-308; Bambra, 1992, Scand. J. Immunol. 11:118-122).

The invention also includes contraceptive methods whereby a modified immunoglobulin of the invention is administered in conjunction with use of another
 5 contraceptive method, such as, but not limited to, barrier methods such as the use of condoms or diaphragms or cervical caps, or intravaginal use of contraceptive compounds such as, but not limited to, non-oxynol-9, intrauterine devices, birth control pills or implants, etc.

The invention also includes administrations of anti-anti-idiotypic antibodies against a
 10 modified immunoglobulin of the invention to acutely neutralize the contraceptive activity of the modified immunoglobulin.

The methods and vaccine compositions of the present invention may be used to elicit a humoral and/or a cell-mediated response against a modified immunoglobulin in a subject. In one specific embodiment, the methods and compositions of the invention elicit a
 15 humoral response in a subject. In another specific embodiment, the methods and compositions of the invention elicit a cell-mediated response in a subject. In a preferred embodiment, the methods and compositions of the invention elicit both a humoral and a cell-mediated response.

The subjects to which the present invention is applicable may be any mammalian or
 20 vertebrate species, which include, but are not limited to, cows, horses, sheep, pigs, fowl (e.g., chickens), goats, cats, dogs, hamsters, mice, rats, monkeys, rabbits, chimpanzees, and humans. In a preferred embodiment, the subject is a human.

25 5.2.1. GENE THERAPY

Gene therapy may be used by administering a nucleic acid containing a nucleotide sequence encoding the modified immunoglobulin of the invention as a contraceptive. In this embodiment of the invention, the therapeutic nucleic acid encodes a sequence that produces intracellularly (without a leader sequence) or intercellularly (with a leader
 30 sequence), a modified immunoglobulin.

For general reviews of the methods of gene therapy, see Goldspiel et al., 1993, *Clinical Pharmacy* 12:488-505; Wu and Wu, 1991, *Biotherapy* 3:87-95; Tolstoshev, 1993, *Ann. Rev. Pharmacol. Toxicol.* 32:573-596; Mulligan, 1993, *Science* 260:926-932; and Morgan and Anderson, 1993, *Ann. Rev. Biochem.* 62:191-217). Methods commonly known
 35 in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, *Current Protocols in Molecular Biology*, John Wiley & Sons, NY; Kriegler,

1990, *Gene Transfer and Expression, A Laboratory Manual*, Stockton Press, NY; and in Chapters 12 and 13, Dracopoli et al. (eds), 1994, *Current Protocols in Human Genetics*, John Wiley & Sons, NY).

In one aspect, the therapeutic nucleic acid comprises an expression vector that
5 expresses the modified immunoglobulin molecule.

Delivery of the nucleic acid into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vector or a delivery complex, or indirect, in which case, cells are first transformed with the nucleic acid *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo*
10 or *ex vivo* gene therapy.

In a specific embodiment, the nucleic acid is directly administered *in vivo*, where it is expressed to produce the antibodies. This can be accomplished by any of numerous methods known in the art, *e.g.*, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by infection
15 using a defective or attenuated retroviral or other viral vector (see U.S. Patent No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in biopolymers (*e.g.*, poly- β -1- \rightarrow 4-N-acetylglucosamine polysaccharide; see U.S. Patent No. 5,635,493), encapsulation in liposomes, microparticles,
20 or microcapsules, or by administering it in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see *e.g.*, Wu and Wu, 1987, *J. Biol. Chem.* 262:4429-4432), etc. In another embodiment, a nucleic acid-ligand complex can be formed in which the ligand comprises a fusogenic viral peptide
25 to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, *e.g.*, PCT Publications WO 92/06180 dated April 16, 1992 (Wu et al.); WO 92/22635 dated December 23, 1992 (Wilson et al.); WO92/20316 dated November 26, 1992 (Findeis et al.); WO93/14188 dated July 22, 1993
30 (Young). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, *Proc. Natl. Acad. Sci. USA* 86:8932-8935; Zijlstra et al., 1989, *Nature* 342:435-438).

Alternatively, single chain antibodies, such as neutralizing antibodies, which bind to intracellular epitopes can also be administered. Such single chain antibodies can be
35 administered, for example, by expressing nucleotide sequences encoding single-chain antibodies within the target cell population by utilizing, for example, techniques such as

those described in Marasco et al. (Marasco et al., 1993, *Proc. Natl. Acad. Sci. USA* 90:7889-7893). Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the
 5 central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, 1993, *Current Opinion in Genetics and Development* 3:499-503 present a review of adenovirus-based gene therapy. Bout et al., 1994, *Human Gene Therapy* 5:3-10 demonstrated the use of
 10 adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld et al., 1991, *Science* 252:431-434; Rosenfeld et al., 1992, *Cell* 68:143-155; and Mastrangeli et al., 1993, *J. Clin. Invest.* 91:225-234. Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al., 1993, *Proc. Soc. Exp. Biol. Med.* 204:289-300).

The form and amount of therapeutic nucleic acid envisioned for use depends on the
 15 type of disease and the severity of its desired effect, patient state, etc., and can be determined by one skilled in the art.

5.3. VACCINE FORMULATIONS AND ADMINISTRATION

The invention also provides vaccine formulations containing Therapeutics of the
 20 invention, which vaccine formulations are suitable for administration to elicit a protective immune (humoral and/or cell mediated) response against certain antigens, e.g., for the contraceptive uses described herein.

Suitable preparations of such vaccines include injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to
 25 injection, may also be prepared. The preparation may also be emulsified, or the polypeptides encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, buffered saline, dextrose, glycerol, ethanol, sterile isotonic aqueous buffer or the like and combinations
 30 thereof. In addition, if desired, the vaccine preparation may also include minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine.

Examples of adjuvants which may be effective, include, but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-
 35 nor-muramyl-L-alanyl-D-isoglutamine, N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine.

The effectiveness of an adjuvant may be determined by measuring the induction of anti-idiotypic antibodies directed against the injected immunoglobulin formulated with the particular adjuvant.

5 The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

10 Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is administered by injection, an ampoule of sterile diluent can be provided so that the ingredients may be mixed prior to administration.

15 In a specific embodiment, the lyophilized modified immunoglobulin of the invention is provided in a first container; a second container comprises diluent consisting of an aqueous solution of 50% glycerin, 0.25% phenol, and an antiseptic (*e.g.*, 0.005% brilliant green).

20 The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the vaccine formulations of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

25 The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Composition comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

30 The subject to which the vaccine is administered is preferably a mammal, most preferably a human, but can also be a non-human animal including but not limited to cows, horses, sheep, pigs, fowl (*e.g.*, chickens), goats, cats, dogs, hamsters, mice and rats.

35 Many methods may be used to introduce the vaccine formulations of the invention; these include but are not limited to oral, intracerebral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal routes, and via scarification

(scratching through the top layers of skin, *e.g.*, using a bifurcated needle) or any other standard routes of immunization. In a specific embodiment, scarification is employed.

The precise dose of the modified immunoglobulin molecule to be employed in the formulation will also depend on the route of administration, and the nature of the patient, and should be decided according to the judgment of the practitioner and each patient's circumstances according to standard clinical techniques. An effective immunizing amount is that amount sufficient to produce an immune response to the modified immunoglobulin molecule in the host (*i.e.*, an anti-idiotypic reaction) to which the vaccine preparation is administered. Effective doses may also be extrapolated from dose-response curves derived from animal model test systems.

5.4. METHOD OF PRODUCING THE MODIFIED IMMUNOGLOBULINS

The modified immunoglobulins of the invention can be produced by any method known in the art for the synthesis of immunoglobulins, in particular, by chemical synthesis or by recombinant expression, and is preferably produced by recombinant expression techniques.

Recombinant expression of the modified immunoglobulin of the invention, or fragment, derivative or analog thereof, requires construction of a nucleic acid that encodes the modified immunoglobulin. If the nucleotide sequence of the modified immunoglobulin is known, a nucleic acid encoding the modified immunoglobulin may be assembled from chemically synthesized oligonucleotides (*e.g.*, as described in Kutmeier et al., 1994, *BioTechniques* 17:242), which, briefly, involves the synthesis of overlapping oligonucleotides containing portions of the sequence encoding the modified immunoglobulin, annealing and ligation of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR, *e.g.*, as exemplified in Section 6, *infra*.

Alternatively, the nucleic acid encoding the modified immunoglobulin may be generated from a nucleic acid encoding the immunoglobulin from which the modified immunoglobulin was derived. If a clone containing the nucleic acid encoding the particular immunoglobulin is not available, but the sequence of the immunoglobulin molecule is known, a nucleic acid encoding the immunoglobulin may be obtained from a suitable source (*e.g.*, an antibody cDNA library, or cDNA library generated from any tissue or cells expressing the immunoglobulin) by PCR amplification using synthetic primers hybridizable to the 3' and 5' ends of the sequence or by hybridization using an oligonucleotide probe specific for the particular gene sequence.

If an immunoglobulin molecule that specifically recognizes a particular antigen is not available (or a source for a cDNA library for cloning a nucleic acid encoding such an

immunoglobulin is not available), immunoglobulins specific for a particular antigen may be generated by any method known in the art, for example, by immunizing an animal, such as a rabbit, to generate polyclonal antibodies or, more preferably, by generating monoclonal antibodies, *e.g.*, as described by Kohler and Milstein (1975, *Nature* 256:495-497) or, as
5 described by Kozbon et al. (1983, *Immunology Today* 4:72) or Cole et al. (1985 in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). Alternatively, a clone encoding at least the Fab portion of the immunoglobulin can be obtained by screening Fab expression libraries (*e.g.*, as described in Huse et al., 1989, *Science* 246:1275-1281) for clones of Fab fragments that bind the specific antigen or by screening
10 antibody libraries (see, *e.g.*, Clackson et al., 1991, *Nature* 352:624; Hane et al., 1997 *Proc. Natl. Acad. Sci. USA* 94:4937).

Once a nucleic acid encoding at least the variable domain of the immunoglobulin molecule is obtained, it may be introduced into any available cloning vector, and may be introduced into a vector containing the nucleotide sequence encoding the constant region of
15 the immunoglobulin molecule (see, *e.g.*, PCT Publication WO 86/05807; PCT Publication WO 89/01036; U.S. Patent No. 5,122,464; and Bebbington, 1991, *Methods in Enzymology* 2:136-145). Vectors containing the complete light or heavy chain for co-expression with the nucleic acid to allow the expression of a complete antibody molecule are also available, see *Id.* Then, the nucleic acid encoding the immunoglobulin can be modified to introduce
20 the nucleotide substitutions or deletion necessary to substitute (or delete) the one or more variable region cysteine residues participating in an intrachain disulfide bond with an amino acid residue that does not contain a sulfhydryl group, along with any other desired amino acid substitutions, deletions or insertions. Such modifications can be carried out by any method known in the art for the introduction of specific mutations or deletions in a
25 nucleotide sequence, for example, but not limited to, chemical mutagenesis, *in vitro* site directed mutagenesis (Hutchinson et al., 1978, *J. Biol. Chem.* 253:6551), PCR based methods, etc.

In addition, techniques developed for the production of chimeric antibodies (Morrison et al., 1984, *Proc. Natl. Acad. Sci.* 81:851-855; Neuberger et al., 1984, *Nature*
30 312:604-608; Takeda et al., 1985, *Nature* 314:452-454) by splicing genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can also be used. As described *supra*, a chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine monoclonal
35 antibody and a constant region derived from a human immunoglobulin, *e.g.*, humanized antibodies.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,694,778; Bird, 1988, *Science* 242:423-42; Huston et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:5879-5883; and Ward et al., 1989, *Nature* 334:544-54) can be adapted to produce single chain antibodies. Single chain antibodies are formed by linking the heavy
5 and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. Techniques for the assembly of functional Fv fragments in *E. coli* may also be used (Skerra et al., 1988, *Science* 242:1038-1041).

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂
10 fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments.

Once a nucleic acid encoding the modified immunoglobulin molecule of the invention has been obtained, the vector for the production of the immunoglobulin molecule
15 may be produced by recombinant DNA technology using techniques well known in the art. The modified immunoglobulin molecule can then be recombinantly expressed and isolated by any method known in the art, for example, using the method described in Section 6, *supra*, (see also Bebbington, 1991, *Methods in Enzymology* 2:136-145). Briefly, COS cells, or any other appropriate cultured cells, can be transiently or non-transiently
20 transfected with the expression vector encoding the modified immunoglobulin, cultured for an appropriate period of time to permit immunoglobulin expression, and then the supernatant can be harvested from the COS cells, which supernatant contains the secreted, expressed modified immunoglobulin.

Methods which are well known to those skilled in the art can be used to construct
25 expression vectors containing the immunoglobulin molecule coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. See, for example, the techniques described in Sambrook et al. (1990, *Molecular Cloning, A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory, Cold
30 Spring Harbor, NY) and Ausubel et al. (eds., 1998, *Current Protocols in Molecular Biology*, John Wiley & Sons, NY).

The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce the immunoglobulin of the invention.

35 The host cells used to express the recombinant antibody of the invention may be either bacterial cells such as *Escherichia coli*, or, preferably, eukaryotic cells, especially for

the expression of whole recombinant immunoglobulin molecules. In particular, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for immunoglobulins (Foecking et al., 198, *Gene* 45:101;

5 Cockett et al., 1990, *Bio/Technology* 8:2).

A variety of host-expression vector systems may be utilized to express the modified immunoglobulin molecules of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the
10 appropriate nucleotide coding sequences, express the immunoglobulin molecule of the invention in situ. These include but are not limited to microorganisms such as bacteria (*e.g.*, *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing immunoglobulin coding sequences; yeast (*e.g.*, *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors
15 containing immunoglobulin coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the immunoglobulin coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing immunoglobulin
20 coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously
25 selected depending upon the use intended for the immunoglobulin molecule being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of an immunoglobulin molecule, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector
30 pUR278 (Ruther et al., 1983, *EMBO J.* 2:1791), in which the immunoglobulin coding sequence may be ligated individually into the vector in frame with the *lac Z* coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, *Nucleic Acids Res.* 13:3101-3109; Van Heeke & Schuster, 1989, *J. Biol. Chem.* 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins
35 with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to a matrix glutathione-

agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

5 In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The immunoglobulin coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).

10 In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the immunoglobulin coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a
15 recombinant virus that is viable and capable of expressing the immunoglobulin molecule in infected hosts (*e.g.*, see Logan & Shenk, 1984, *Proc. Natl. Acad. Sci. USA* 81:355-359). Specific initiation signals may also be required for efficient translation of inserted immunoglobulin coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading
20 frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, *Methods in Enzymol.* 153:51-544).

25 In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of
30 proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS,
35 MDCK, 293, 3T3, WI38.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the immunoglobulin molecule may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the immunoglobulin molecule. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the immunoglobulin molecule.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., 1977, *Cell* 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1972, *Proc. Natl. Acad. Sci. USA* 48:202), and adenine phosphoribosyltransferase (Lowy et al., 1980, *Cell* 22:817) genes can be employed in tk⁻, hgprt⁻ or aprt⁻ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al., 1980, *Natl. Acad. Sci. USA* 77:357; O'Hare et al., 1981, *Proc. Natl. Acad. Sci. USA* 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072); neo, which confers resistance to the aminoglycoside G-418 (*Clinical Pharmacy* 12:488-505; Wu and Wu, 1991, *Biotherapy* 3:87-95; Tolstoshev, 1993, *Ann. Rev. Pharmacol. Toxicol.* 32:573-596; Mulligan, 1993, *Science* 260:926-932; and Morgan and Anderson, 1993, *Ann. Rev. Biochem.* 62:191-217; May, 1993, *TIBTECH* 11(5):155-215). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, *Current Protocols in Molecular Biology*, John Wiley & Sons, NY; Kriegler, 1990, *Gene Transfer and Expression, A Laboratory Manual*, Stockton Press, NY; and in Chapters 12 and 13, Dracopoli et al. (eds), 1994, *Current Protocols in Human Genetics*, John Wiley & Sons, NY.; Colberre-Garapin et al., 1981, *J. Mol. Biol.* 150:1.

Alternatively, any fusion protein may be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht et al., 1991, *Proc. Natl. Acad. Sci. USA* 88:8972-897). In

this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the open reading frame of the gene is translationally fused to an amino-terminal tag consisting of six histidine residues. The tag serves as a matrix binding domain for the fusion protein. Extracts from cells infected with recombinant vaccinia virus are loaded onto
 5 Ni^{2+} -nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

The expression levels of the immunoglobulin molecule can be increased by vector amplification (for a review, see Bebbington and Hentschel, *the Use of Vectors Based on Gene Amplification for the Expression of Cloned Genes in Mammalian Cells in DNA*
 10 *Cloning*, Vol.3. (Academic Press, New York, 1987)). When a marker in the vector system expressing immunoglobulin is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the immunoglobulin gene, production of the immunoglobulin will also increase (Crouse et al., 1983, *Mol. Cell. Biol.* 3:257).

15 The host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes both heavy and light chain polypeptides. In such
 20 situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot, 1986, *Nature* 322:52; Kohler, 1980, *Proc. Natl. Acad. Sci. USA* 77:2197). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

Once the modified immunoglobulin molecule of the invention has been
 25 recombinantly expressed, it may be purified by any method known in the art for purification of an immunoglobulin molecule, for example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

30

5.5. DEMONSTRATION OF THERAPEUTIC UTILITY

The modified antibodies of the invention can be screened or assayed in a variety of ways for efficacy in treating or preventing a particular disease .

First, the immunopotency of a vaccine formulation containing the modified antibody
 35 of the invention can be determined by monitoring the anti-idiotypic response of test animals following immunization with the vaccine. Generation of a humoral response may be taken

5

10

15

30

Additionally, the modified antibodies of the invention may also be tested directly *in vivo*. The strength of the immune response *in vivo* to the modified immunoglobulin may be determined by any method known in the art, for example, but not limited to, delayed hypersensitivity skin tests and assays of the activity of cytolytic T-lymphocytes *in vitro*.

5 Delayed hypersensitivity skin tests are of great value in the testing of the overall immunocompetence and cellular immunity to an antigen. Proper technique of skin testing requires that the antigens be stored sterile at 4°C, protected from light and reconstituted shortly before use. A 25- or 27-gauge needle ensures intradermal, rather than subcutaneous, administration of antigen. Twenty-four and 48 hours after intradermal administration of the
10 antigen, the largest dimensions of both erythema and induration are measured with a ruler. Hypoactivity to any given antigen or group of antigens is confirmed by testing with higher concentrations of antigen or, in ambiguous circumstances, by a repeat test with an intermediate test.

To test the activity of cytolytic T-lymphocytes, T-lymphocytes isolated from the
15 immunized subject, *e.g.*, by the Ficoll-Hypaque centrifugation gradient technique, are restimulated with cells bearing the antigen against which the modified antibody was directed in 3 ml RPMI medium containing 10% fetal calf serum. In some experiments, 33% secondary mixed lymphocyte culture supernatant or IL-2 is included in the culture medium as a source of T cell growth factors. In order to measure the primary response of cytolytic
20 T-lymphocytes after immunization, the isolated T cells are cultured with or without the cells bearing the antigen. After six days, the cultures are tested for cytotoxicity in a 4 hour ⁵¹Cr-release assay. The spontaneous ⁵¹Cr-release of the targets should reach a level less than 20% if immunization was effective (Heike et al., *J. Immunotherapy* 15:15-174).

The efficacy of the modified antibody as a contraceptive can also be tested by any
25 method known for tested contraceptive methods. For example, a vaccine composition containing a modified antibody of the invention specific for an antigen of a protein or cell involved in reproductive function. First, the level of the particular antigen in the subject can be measured by any method known in the art where a reduction in the level of the antigen compared to the level prior to administration of the modified antibody (accounting for
30 normal, cyclical changes of the level of the particular antigen) indicates that the modified antibody may be effective. The modified antibody must then be administered to a population of child bearing age (and having partners of childbearing age) and the percentage of females that conceive over a suitable period of time is determined. If the number of females that conceive is significantly lower than those in a control population, *e.g.*, those
35 administered a placebo or not using a contraceptive method, indicates that the modified antibody is effective as a contraceptive.

Additionally, the efficacy of the contraceptive vaccine may be assayed by administering the vaccine to a subject or animal model, allowing an appropriate amount of time for the production of anti-idiotypic antibodies, and then testing serum taken from the subject or animal for the ability to bind the particular antigen (indicating that an anti-idiotypic reaction has occurred) and/or testing whether the serum can block fertilization in vitro, which can be tested by any method known in the art, for example as described in Brannen-Brock et al., 1985, Arch. Androl. 15:15-19. . .

6. **EXAMPLE: ANTI-IDIOTYPIC VACCINE INDUCER FOR COLON**
CANCER

This example describes the construction of a modified antibody derived from the monoclonal antibody Mab31.1 (hybridoma secreting Mab31.1 is available from the American Type Tissue Collection as accession No. HB12314). Mab31.1 recognizes an antigen expressed by human colon carcinomas. The modified antibody of the invention, based on Mab31.1, was engineered to have variable region cysteine residues of both the heavy and light chain variable regions substituted with alanine. Therefore, the resulting modified antibody, was missing intrachain disulfide bonds in either the heavy and light chain variable regions.

6.1. **CONSTRUCTION OF A MODIFIED ANTIBODY**

The strategy for construction of the modified antibody was to construct two engineered genes that encoded the heavy and light chain variable regions wherein specific cysteine residues, known to be important in intra-chain disulfide bonding , were altered to alanine. Alanine residues were substituted for the cysteine residues at positions 22 and 92 of the heavy chain variable region of the antibody derived from Mab31.1 or at positions 23 and 88 of the Mab31.1 light chain variable region of the antibody derived from Mab31.1. In order to construct these engineered genes, groups of oligonucleotides were assembled (as discussed below) and inserted into an appropriate vector providing constant regions.

In order to construct variable region genes encoding CDRs lacking intrachain disulfide bonds, the following strategy was performed.

First, single strand oligonucleotides were annealed to create cohesive double stranded DNA fragments (as diagramed in Figure 8, Step 1). Specifically, oligonucleotides of about 80 bases in length corresponding to the sequences of interest with 20 base overlapping regions were synthesized using automated techniques of GenoSys Biotech Inc. The specific sequences of each of these oligonucleotides. The specific sequences of these

oligonucleotides are presented in Figures 16A and 16B. Figure 16A list the group of ten oligos used in engineering a heavy chain variable region gene called 2CAVHCOL1. 2CAVHCOL1 lacked 2 cysteine residues as compared to the consensus heavy chain variable gene. Figure 16B lists the group of 12 oligos used in the engineering of the light chain variable region gene called 2CAVLCOL1. 2CAVLCOL1 lacked two cysteine residues as compared to the consensus light chain variable region gene. In order to combine the oligos into the desired gene, groups of 10 or 12 oligos were combined as described below and as presented in Figure 8, where the identities of oligos 1 to 10 indicated in Figure 8 are provided in Table 5. Prior to combining, each oligonucleotide was 5' phosphorylated as follows: 25µl of each oligo was incubated for 1 hour in the presence of T4 polynucleotide kinase and 50mM ATP at 37°C. The reactions were stopped by heating for 5 minutes at 70°C followed by ethanol precipitation. Once phosphorylated, complementary oligonucleotides (oligo 1 + oligo 10, oligo 2 + oligo 9, oligo 3 + oligo 8, oligo 4 + oligo 7, oligo 5 + oligo 6), as shown in Figure 8, were then mixed in sterile microcentrifuge tubes and annealed by heating the tube in a water bath at 65°C for 5 minutes followed by cooling at room temperature for 30 minutes. Annealing resulted in short double strand DNA fragments with cohesive ends.

Next, the cohesive double stand DNA fragments were ligated into longer strands (Figure 8, Steps 2-4), until the engineered variable region gene was assembled. Specifically, cohesive double strand DNA fragments were ligated in the presence of T4 DNA ligase and 10mM ATP for 2 hours in a water bath maintained at 16°C. Annealed oligo 1/10 was mixed with annealed oligo 2/9, and annealed oligo 3/8 was mixed with annealed oligo 4/7. The resulting oligos were labeled oligo 1/10/2/9 and oligo 3/8/4/7. Next, oligo 3/8/4/7 was ligated to oligo 5/6. The resulting oligo 3/8/4/7/5/6 was then ligated to oligo 1/10/2/9 resulted in a full length variable region gene.

Alternatively, when groups of 12 oligos were used, the order of addition was: 1+12 = 1/12, 2+11=2/11, 3+10=3/10, 4+9=4/9, 5+8=5/8, 6+7=6/7, 1/12+2/11=1/12/2/11, 3/10+4/9=3/10/4/9, 5/8+6/7=5/8/6/7, 1/12/2/11+3/10/4/9 = 1/12/2/11/3/10/4/9, 1/12/2/11/3/10/4/9+5/8/6/7= full length variable region gene. The names of oligonucleotides used in construction of the engineered genes are listed in Table 5. The modified heavy chain variable region gene was denoted as 2CAVHCOL1. The modified light chain variable region gene was denoted as 2CAVLCOL1.

The resulting modified variable region genes were then purified by gel electrophoresis. To remove unligated excess of oligos and other incomplete DNA fragments, ligated product was run on 1% low melting agarose gel at constant 110 V for 2 hours. The major band containing full length DNA product was cut out and placed in a

sterile 1.5 ml centrifuge tube. To release the DNA from the agarose, the gel slice was digested with f3-Agrase I at 40°C for 3 hours. The DNA was recovered by precipitation with 0.3 M NaOAc and isopropanol at -20°C for 1 hour followed by centrifugation at 12,000 rpm for 15 minutes. The purified DNA pellet was resuspended in 50 µl of TE buffer, pH 8.0. The engineered variable region gene was then amplified by PCR. Specifically, 100 ng of the engineered variable region gene was mixed with 25mM dNTPs, 200 ng of primers and 5 U of high fidelity thermostable Pfu DNA polymerase in buffer. Resulting PCR product was analyzed on 1% agarose gel.

Each purified DNA corresponding to the engineered variable region gene was subsequently inserted into the pUC19 bacterial vector. pUC19, is a 2686 base pair, a high copy number *E. coli* plasmid vector containing a 54 base pair polylinker cloning site in lacZ and an Amp selection marker. In order to prepare the vector for insertion of the engineered variable region gene, 10µg of pUC19 was linearized with *Hinc II* (50 U) for 3 hours at 37°C resulting in a vector with blunt end sequence 5' GTC. To prevent self re-ligation, linear vector DNA was dephosphorylated with 25 U of calf intestine alkaline phosphatase (CIP) for 1 hour at 37°C. In order to insert the engineered variable region gene into the pUC19 vector, approximately 0.5 µg of dephosphorylated linear vector DNA was mixed with 3 µg of phosphorylated variable region gene in the presence of T4 DNA ligase (1000 U), and incubated at 16°C for 12 hours.

The bacterial vector containing the engineered variable region gene was then used to transform bacterial cells. Specifically, freshly prepared competent DH5-α cells, 50 µl, were mixed with 1 µg of pUC19 containing the engineered variable region gene and transferred to an electroporation cuvette (0.2 cm gap; Bio-Rad). Each cuvette was pulsed at 2.5 kV/200 ohm/25 µF in an electroporator (Bio-Rad Gene Pulser). Immediately thereafter, 1 ml of SOC media was added to each cuvette and cells were allowed to recover for 1 hour at 37°C in centrifuge tubes. An aliquot of cells from each transformation was removed, diluted 1:100, then 100 µl plated onto LB plates containing ampicillin (Amp 40 µg/ml). The plates were incubated at 37°C overnight due to the presence of the Amp marker. Only transformants containing pUC19 vector grew on LB/Amp plates.

A single transformant colony was picked and grown overnight in a 3 ml LB/Amp sterile glass tube with constant shaking at 37°C. The plasmid DNA was isolated using Easy Prep columns (Pharmacia Biotech.) and suspended in 100 µl of TE buffer, pH 7.5. To confirm the presence of gene insert in pUC19, 25 µl of plasmid DNA from each colony was digested with a restriction endonuclease for 1 hour at 37°C, and was analyzed on a 1% agarose gel. By this method plasmid DNA containing gene insert was resistant to enzyme cleavage due to loss of restriction site (5'..GTCGAC.. 3') and migrated as closed circular

(CC) DNA, while those plasmids without insert were cleaved and migrated as linear (L) double strand DNA fragment on gel.

Sub BB In order to confirm correct gene sequences of the engineered variable region genes and to eliminate the possibility of unwanted mutations generated during the construction procedure, DNA sequencing was performed using M13/pUC reverse primer (5'AACAGCTATGACCATG 3') for the clones as well as PCR gene products using 5' end 20 base primer (5' GAATT CATGGCTTG GGTGTG 3') on automated ABI 377 DNA Sequencer. All clones were confirmed to contain correct sequences.

10 **Table 5. Construction of gene encoding modified antibodies containing CDRs from Mab 31.1**

	Oligo1	Oligo 2	Oligo 3	Oligo 4	Oligo 5	Oligo 6	Oligo 7	Oligo 8	Oligo 9	Oligo10
2CAVHC	VHC1	VHC2	VHC3	VHC4	VHC5	VHC	VHC7	VHC8	VHC9	VHC10
OLI										
2CAVLC	VLC1	VLC2	VLC3	VLC4	VLC5	VLC	VLC7	VLC8	VLC9	VLC10
OLI										

6.3. INSERTION OF THE ENGINEERED VARIABLE REGION GENE INTO A MAMMALIAN EXPRESSION VECTOR

A complete antibody light chain has both a variable region and a constant region. A complete antibody heavy chain contains a variable region, a constant region, and a hinge region. A modified variable region genes 2CAVHCOL1 or 2CAVLCOL1 were inserted into vectors containing appropriate constant regions. Engineered variable region genes lacking cysteine residues in the light chain, were inserted into the pMRRO10.1 vector Figure 6A. The pMRRO10.1 vector contained a human kappa light chain constant region. Insertion of the engineered light chain variable region into this vector gave a complete light chain sequence. Alternatively, the engineered variable region gene lacking cysteine residues in the heavy chain, were inserted into the pGAMMA1 vector Figure 6B. The pGAMMA1 vector contained human and IgG1 constant region and hinge region sequences. Insertion of the engineered heavy chain variable region gene into this vector gave a complete heavy chain sequence.

In order to engineer a mammalian vector comprising both heavy chain and light chain genes, the complete light chain sequence and complete heavy chain sequence were inserted into mammalian expression vector pNEPuDGV as shown in Figure 6C (Bebbington, C.R., 1991, In METHODS: A Companion to Methods in Enzymology, vol. 2, pp. 136-145). The resulting vector encoding both light chain and the heavy chain of the modified antibody.

6.4. EXPRESSION OF SYNTHETIC MODIFIED ANTIBODIES IN MAMMALIAN CELLS

To examine the production of assembled antibodies the mammalian expression vector was transfected into COS cells. COS cells (an African green monkey kidney cell line, CV-1, transformed with an origin-defective SV40 virus) were used for short-term transient expression of the synthetic antibodies because of their capacity to replicate circular plasmids containing an SV40 origin of replication to very high copy number. The antibody expression vector was transferred to COS7 cells (obtained from the American Type Culture Collection). The transfected cells were grown in Dulbecco's modified Eagle's Medium and transfected with the expression vectors using calcium precipitation (Sullivan et al., *FEBS Lett.* 285:120-123, 1991). The transfected cells were cultured for 72 hours after which supernatants were collected. Supernatants from transfected COS cells were assayed using ELISA method for assembled IgG. ELISA involves capture of the samples and standards onto a 96-well plate coated with an anti-human IgG Fc. Bound assembled IgG was detected with an anti-human Kappa chain linked to horse radish peroxidase (HRP) and the substrate tetramethylbenzidine (TMB). Color development was proportional to the amount of assembled antibody present in the sample.

6.5. MODIFIED ANTIBODY IMMUNOSPECIFICALLY BINDS TO HUMAN COLON CARCINOMA CELLS AND ANTIGENS PRODUCED BY THESE CELLS

The modified antibody was expressed and isolated as indicated in Section 6.4, *supra*. The binding capacity and specificity were then assayed using LS-174T cells WiDR cells (a human colon cancer cell line) and an antigen derived from these cells.

In order to examine the binding potency as well as specificity of MA31.1 binding, a dot blot analysis was performed (see Figure 9). Membrane preparations from LS-174T cells was applied to a nitrocellulose membrane using a Bio-Blot apparatus (Bio-Rad). The wells were blocked for non-specific binding using skim milk. Biotinylated antibody derived from Mab31.1 was incubated in all wells. Unlabelled antibody at concentrations of 0.003 to 20 nM was then applied to the nitrocellulose membrane and allowed to incubate. Unbound antibody was removed from the membrane by washing and a second antibody, alkaline phosphatase labeled antihuman IgG, was added. Finally, an alkaline phosphatase substrate was added which generated a dark purple precipitate, indicating the presence of bound labeled antibody. Figure 9 provides the results of the dot blot analysis. The figure demonstrated that the labeled antibody bound to the LS-174 T cells. Additionally, the unlabeled antibody competed with biotinylated antibody binding, indicating specificity of binding of the antibody derived from Mab31.1 to tumor cell antigens.

6.6. ANTI-IDIOTYPE RESPONSE

The effect on binding of modified antibody to LS-174T cells was examined in a competition binding assay. LS-174T cells are human colon carcinoma cells which express antigen recognized by the Mab31.1 antibody. Peptides containing the sequence of one of the CDRs of the Mab31.1 antibody were generated. These peptides, the modified antibody and the control antibody derived from Mab31.1 were administered to mice in order to generate antisera against the CDR regions of Mab31.1 and the antibodies. Blood samples from mice were drawn two weeks and four weeks following injection. Antisera from the immunized mice were used in binding competition assays presented in Figures 10A and B.

Antisera and biotinylated antibodies were assayed for their ability to bind LS-174T cells. As demonstrated in Figure 10A and B, antisera raised to the CDR3 and CDR4 peptides dramatically competed for control antibody (antibody derived from Mab31.1) binding to LS-174T cells. Additionally, antisera raised against CDR1 and CDR2 also significantly competed for control antibody binding to LS-174T cells. Additionally, antisera from mice injected with the 2CAVHCOL1 and 2CAVLCOL1 antibodies (*i.e.*, the modified antibodies having the cysteine to alanine change in the variable region) competed for binding with the biotinylated antibody derived from Mab31.1 better than antiserum from mice injected with the antibody derived from Mab31.1 (Figure 10B). This result indicates that administration of the antibodies having the cysteine to alanine change in the variable region elicit an anti-idiotypic antibodies that recognize the colon carcinoma cell antigen better than antibodies with variable region intra-chain disulfide bonds.

Table 6. Biotin-Labeled Peptides Derived from CDR Sequences of Mab 31.1

Sub B9

Peptide ID	Sequence
COL311 L1	biotin-N-Thr-Ala-Lys-Ala-Ser-Gln-Ser-Val-Ser-Asn-Asp-Val-Ala
COL311 L2	biotin-N-Ile-Tyr-Tyr-Ala-Ser-Asn-Arg-Tyr-Thr
COL311 L3	biotin-N-Phe-Ala-Gln-Gln-Asp-Tyr-Ser-Ser-Pro-Leu-Thr
COL311 H1	biotin-N-Phe-Thr-Asn-Tyr-Gly-Met-Asn
COL311 H2	biotin-N-Ala-Gly-Trp-Ile-Asn-Thr-Tyr-Thr-Gly-Glu-Pro-Thr-Tyr-Ala-Asp-Asp-Phe-Lys-Gly
COL311 H3	biotin-N-Ala-Arg-Ala-Tyr-Tyr-Gly-Lys-Tyr-Phe-Asp-Tyr

7. EXAMPLE: SPERM ANTIGEN VACCINES

The example herein describes the construction of defined epitopes that replace the complementarity determining regions (CDR) of an antibody. Specifically, the

epitopes are derived from sperm antigens SP-10, LDH-C₄ or MSA-63. These constructs express an antibody, which, when injected into an appropriate host, induces an immune reaction that precipitates the formation of anti-idiotypic antibodies that are active against the sperm antigens.

- 5 The strategy for producing the antibody containing a sperm cell epitope is outlined as follows: (1) a CDR is engineered to contain a nucleotide sequence encoding one or more epitopes from a sperm specific protein, (2) the engineered CDR is then cloned into a mammalian expression vector containing the appropriate heavy or light chain constant regions, (3) the vector is transfected into a cell that supports expression, proper folding and
- 10 modification of functional antibodies, (4) the antibody is harvested from the supernatant and is confirmed for the epitope expression by standard assays (e.g. ELISA, western blot, etc.), and (5) the antibody is used as an immunogen in an appropriate host to generate anti-sperm antibodies, thereby inducing long lasting infertility.

15 7.1. CONSTRUCTION OF THE SPERM ANTIGEN VACCINE

The following describes the construction of a modified variable region gene containing at least one CDR that contains a sperm antigen epitope, *i.e.*, SP-10 or LDH-C₄ epitope and/or an MSA-63 epitope.

- First, an epitope is chosen and defined so that oligonucleotides may be
- 20 synthesized. In the following example, an SP-10 epitope from the sperm antigen SP-10 is used. SP-10 is a suitable epitope because it is expressed exclusively in sperm cells. It is also expressed on the surface of the membrane of the acrosome, thus, it is accessible to therapeutic antibodies. Other antibodies are produced that contain portions of the LDH-C₄ and MSA-63 antigens.

- 25 The nucleotide and protein sequences of the SP-10 epitope are:

GAA TTC CAG CCT TCA GGT GAA CAT GGC TCC GGT GAA CAG CCT TCT GGT
GAG CAG GCC TCG GGT GAA CAG CCT TCA GGT GAG CAC GCT TCA GGG GAA
CAG GCT TCA GGT GCA CCA ATT TCA AGC ACA TCT ACA GGC ACA ATA TTA
AAT TGC TAC ACA TGT GCT TAT ATG AAT GAT CAA GGA AAA TGT CTT CGT
GGA GAG GGA ACC TGC ATC ACT CAG AAT TC;

- 30 Gln Pro Ser Gly Glu His Gly Glu Gln Pro Ser Gly Glu Gln Ala Ser Gly Glu Gln Pro Ser gly
Glu His Ala Ser Gly Glu Gln Ala Ser Gly Ala Gln Ile Ser Ser Thr Ser Thr Gly Thr Ile Leu
Asn Cys Tyr Thr Cys Ala Tyr Met Asn Asp Gln Gly Lys Cys Leu Arg Gly Glu Gly Thr
Cys Ile Thr Gln Asn.

35 The replacement of an antibody's CDR with another epitope is made easier by the fact that the variable region sequence of antibodies are relatively short, and are known. One is then able to synthetically generate a series of complementary oligonucleotides that, when annealed and ligated, reconstruct the entire coding region of

variable region portion of the gene. In this manner, the CDR is replaced with sequences of the epitope of interest, in this example, SP-10. The following is a list of the sequences of the oligonucleotides designed for cloning SP-10 epitopes into the CDR:

- Oligo SP 1:**
 5 GAA TTC CAG CCT TCA GGT GAA CAT GGC TCC GGT GAA CAG CCT TCT GGT
 GAG CAG GCC TCG GGT GAA CAG CCT TAG,
Oligo SP 2:
 GTG AGC ACG CTT CAG GGG AAC AGC CTT CAG GTG CAC CAA TTT CAA GCA
 CAT CTA CAG GCA CAA TAT TAA ATT GCT,
Oligo SP 3:
 10 ACA CAT GTG CTT ATA TGA ATG ATC AAG GAA AAT GTC TTC GTG GAG AGG
 GAA CCT GCA TCA CTC AGA ATT C,
Oligo SP 3a(3Cys->Ala):
 ACA CAG CAG CTT ATA TGA ATG ATC AAG GAA AAG CAC TTC GTG GAG AGG
 GAA CCG CAA TCA CTC AGA ATT C,
Oligo SP 4:
 GAA TTC TGA GTG ATG CAG GTT CCC TCT CCA CGA AGA CAT TTT CCT TGA
 TCA TTC ATA TAA GCA CAT GTG TAG CAA TTT A,
Oligo SP 4a (3Cys->Ala):
 15 GAA TTC TGA GTG ATT GCG GTT CCC TCT CCA CGA AGT GCT TTT TGA TGA
 TCA TTC ATA TAA GCT GCT GTG TAG CAA TTT A,
Oligo SP 5:
 ATA TTG TGC CTG TAG ATG TGC TTG AAA TTG GTG CAC CTG AAG CCT GTT
 CCC CTG AAG CGT GCT CAC CTG AAG GCT,
Oligo SP 6:
 20 GTT CTC CCG AGG CCT GCT CAC CAG AAG GCT GTT CAC CGG AGC CAT GTT
 CAC CTG AAG GCT GGA ATT C.

Antibodies containing portions of the MSA-63 antigen are also described.

To identify the optimal portion of the antigen to be introduced into the antibody, oligonucleotides encoding different portions of the antigen are synthesized.

25 *Sub B12* Practically, the first two amino acid codons of the sperm cell specific epitope, MSA-63, an oligonucleotide encoding residues 143 and 144 (i.e. GTC GGC, *infra*), is cloned into the immunoglobulin CDR, using the methods described *infra*. The MSA-63 DNA sequence encoding the epitope:

- 30 GTC GGC AGC CTC CGA AGC AGC CCG CTC CAG AGC CCG CTG CTC CGA CCG
 CTC GTC CAG AGC AGC CTC TGC TTG CTG TTC CTC TTG CTG CGA TAC AGC
 TGC GGC GAC GGC AGC TGC AGC CGA CGA TAC TGC GAC TTG ACG GTG TGC
 CGG CGA ATG TAC TTG CTG CTG CGA TTC ACG GAC CCG CCG CTC CCG CAG
 ACG TGC TGC GTC TTG AGC

The MSA-63 protein sequence epitope encoded by the nucleic acid sequence above, which starts at amino acid 143 and ends at 233.

- 35 Gln Pro Ser Glu Ala Ser Ser Gly Glu Val Ser Gly Asp Glu Ala Gly Glu Gln Val Ser Ser
 Glu Thr Asn Asp Lys Glu Asn Asp Ala Met Ser Thr Pro Leu Pro Ser Thr Ser Ala Ala Ile

Thr Leu Asn Cys His Thr Cys Ala Tyr Met Asn Asp Asp Ala Lys Cys Leu Arg Gly Glu
Gly Val Cys Thr Thr Gln Asn Ser

For the second two amino acid codons, an oligonucleotide encoding residues 144 and 145 is utilized (*i.e.*, GGC AGC). For the third, 145 and 146 and so on until the entire epitope is synthesized and inserted into the CDR, two amino acids at a time. For peptides three amino acids in length, an oligonucleotide encoding residues 143 to 146 is synthesized. The second oligonucleotide synthesized encodes residues 146 to 148. The third encodes residues 148 to 150, and this continues until the entire epitope is covered in this fashion. The next oligonucleotide that is synthesized is four amino acid codons in length. It begins with residues 143 to 146, its second segment is equivalent to residues 145 to 148, its third segment corresponds to residues 147 to 150, and so on until the entire epitope is transitioned in this fashion. The next oligonucleotide synthesized contains five amino acid codons with two overlapping with the previous. For example, the first oligonucleotide encodes residues 143 to 147, and the second residues 146 to 150. This pattern continues until the entire epitope has been transitioned. The next construct encoding an epitope uses nucleotides for six amino acid codons with two overlapping with the previous codons as described *infra*.

The epitopes thereafter contain peptides of seven residues with three overlapping. The pattern of adding one amino acid to each small peptide and increasing the overlap by one codon continues until an overlap of five is reached and then the small peptides are synthesized adding one codon each time until the full length of the epitope is encoded in the CDR. The overlap is never bigger than five amino acid codons although the entire peptide is lengthened by one amino acid in each new combination.

In a specific example, oligomers have been designed which scan the entire length of the MSA-63 epitope and encode 15 amino acids. Each oligo overlaps with the previous one for the equivalent of five amino acids. MSA-63 oligos encoding 15 amino acids, with overlap of five amino acids each:

MSA1: GTC GGC AGC CTC CGA AGC AGC CCG CTC CAG AGC CCG CTG CTC CGA
MSA2: AGC CCG CTG CTC CGA CCG CTC GTC CAG AGC AGC CTC TGC TTG CTG
MSA3: AGC CTC TGC TTG CTG TTC CTC TTG CTG CGA TAC AGC TGC GGC GAC
MSA4: TAC AGC TGC GGC GAC GGC AGC TGC AGC CGA CGA TAC TGC GAC TTG
MSA5: CGA TAC TGC GAC TTG ACG GTG TGC ACG CGA ATG TAC TTG CTG CTG
MSA6: ATG TAC TTG CTG CTG CGA TTC ACG GAC GCG CCG CTC CCG CAG ACG
MSA7: CGA TTC ACG GAC GCG CCG CTC CCG CAG ACG TGC TGC GTC TTG AGC

Sub B14

Antibodies in which a portion of the MSA-63 antigen has been inserted into CDR1, *i.e.*, where residues Lys24 through Ala34 of the consensus contraceptive light chain, the sequence of which is in Figure 15, are replaced with the sequence Gln-Pro-Ser-Glu-Ala-Ser-Ser-Gly-Glu-Val-Ser-Gly-Asp-Glu-Ala-Gly-Glu. The antibody, MSA1, can be

5 constructed using the oligonucleotides provided in Figure 11 in the scheme provided in Figure 8 and described below, where the identities of oligonucleotides 1-12 are indicated in Table 7. The antibody MSA1VAC can also be constructed using the oligos of Figure 11 by the scheme of Figure 8, as indicated in Table 7. MSA1VAC is the same as MSA1 except that the cysteine at position 23 of the light chain variable region has been replaced with

10 alanine. These light chains can be expressed with the heavy chain consensus sequence CONVH1, the sequence of which is provided in Figure 7B, and the construction of which can be accomplished with the oligonucleotides as indicated in Table 4. These single stranded oligonucleotides sequences are annealed to create cohesive double stranded DNA fragments suitable for ligation as diagramed in Figure 8, along with oligonucleotides

15 encoding the remainder of the consensus variable region, to construct the variable region gene. For the MSA-63 containing variable regions MSA1 and MSA1VAC the oligonucleotides corresponding to oligonucleotides 1 to 10, or 1 to 12, of Figure 8 are provided in Table 7, and the sequences of these oligonucleotides are provided in Figure 11. Specifically, oligonucleotides of about 70 bases in length corresponding to the sequences of

20 interest with 20 base overlapping regions are synthesized (GenoSys Biotech Inc.). Each oligonucleotide is 5' phosphorylated as follows: 25 μ l of each oligo is incubated for one hour in the presence of T_4 polynucleotide kinase and 50 mM ATP in appropriate buffer at 37°C. The enzyme is heat killed and the reaction stopped by heating for ten minutes at 70°C followed by ethanol precipitation with sodium acetate. The oligos are then resuspended in

25 TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA).

Complementary oligonucleotides (oligo 1 + oligo 10, oligo 2 + oligo 9, oligo 3 + oligo 8, oligo 4 + oligo 7, and oligo 5 + oligo 6) were then mixed in a sterile microcentrifuge tube and annealed by heating the tube in a water bath at 65°C for 5 minutes followed by cooling at room temperature for 30 minutes. Annealing results in double

30 stranded DNA with cohesive ends. The cohesive double stranded DNA fragments are ligated into longer strands (Figure 8, Steps 2-4), until the engineered variable region gene was assembled. Specifically, cohesive double stranded DNA fragments are ligated in the presence of T_4 DNA ligase, ligase buffer and 10 mM ATP for two hours in a water bath maintained at 16°C. Annealed oligo 1/10 is mixed with annealed oligo 2/9, and annealed

35 oligo 3/8 is mixed with annealed oligo 4/7. The resulting oligos are 1/10/2/9 and 3/8/4/7. Next, oligo 3/8/4/7 is ligated to oligo 5/6. The resulting oligo 3/8/4/7/5/6 is then ligated to

oligo 1/10/2/9 resulting in a full length variable region gene. Alternatively, when 12 oligos are used, the order of addition is $1+12=1/12$, $2+11=2/11$, $3+10=3/10$, $4+9=4/9$, $5+8=5/8$, $6+7=6/7$, $1/12+2/11=1/12/2/11$, $3/10+4/9=3/10/4/9$, $5/8+6/7=5/8/6/7$, $1/12/2/11+3/10/4/9=1/12/2/11/3/10/4/9$,

- 5 $1/12/2/11/3/10/4/9+5/8/6/7=1/12/2/11/3/10/4/9/5/8/6/7$, which is the full length modified variable region gene. The names of oligonucleotides used for construction are listed in Table 7 and Figures 9, , 11, 12C, or 13C.

- Using this method, variable region sequences in which an alanine has been substituted for a cysteine that forms an intrachain disulfide bond can be constructed using
10 oligonucleotides introducing this change. For example, in constructing the antibody contains the SP-10 portion, oligos SP 3a and SP 4a could be used instead of oligo SP3 or SP4.

- The modified variable region DNA fragment is then cloned into a shuttle vector (e.g. pUC19, *infra*) for sequence analysis and upon sequence confirmation, cloned
15 into an expression vector. After running the DNA for two hours at 110 volts in a 1% low melting agarose gel, DNA fragments are visualized by ethidium bromide staining and gel slices are cut out and placed in a sterile microfuge tube. Gel purification removes excess free oligomers that may interfere with future ligations. The DNA is eluted from the agarose by addition with f3-Agrase I at 40°C for three hours. DNA is precipitated using 0.3 M
20 sodium acetate and isopropanol at -20°C for one hour, followed by centrifugation at high speed in a microcentrifuge for ten minutes. Isopropanol is aspirated and the pellet is washed once with 70% ethanol, the sample is spun again and the ethanol is aspirated and the pellet air dried. The DNA pellet is quantitated by running a small fraction of the resuspended pellet (i.e. 1/10th) on a gel and visually comparing to known DNA standards,
25 or measuring the absorbance of UV light at 260 nM. If the quantity of DNA is to limiting for cloning at this point, it can be amplified by PCR techniques well known to those skilled in the art.

7.2 LIGATION OF THE MODIFIED CDR INTO PUC19

- 30 Purified DNA corresponding to the engineered variable region gene is subsequently inserted into the pUC19 vector by ligation. The pUC19 vector is a 2686 base

Table 7

	Oligo 1	Oligo 2	Oligo 3	Oligo 4	Oligo 5	Oligo 6	Oligo 7	Oligo 8	Oligo 9	Oligo 10	Oligo 11	Oligo 12
MSA 1	LDR	DSABL-1	MSAL- CDR1-1	HMVL3	HMVL4	HMVL5	HMVL6	HMVL7	HMVL8	MSAL- CDR1-1c	DSABL-1c	ANTIL DR
MSA1VA C	LDR	DSABL-1	MSALVA C-CDR1- 1	HMVL3	HMVL4	HMVL5	HMVL6	HMVL7	HMVL8	MSALVAC -CDR1-1	DSABL-1c	ANTIL DR
ConVH1	BKHC1	BKHC2	BKHC3	BKHC4	BKHC5	BKHC6	BKHC7	BKHC8	BKHC9	BKHC10		

pair, high copy number *E. coli* plasmid containing a 54 base pair polylinker cloning site in the middle of the *lacZ* gene. The pUC19 vector also contains an ampicillin resistance marker for selection of bacteria containing the plasmid. The pUC19 is digested with the restriction enzyme *Hinc II* (10 µg plasmid in 50 units enzyme). The resulting blunt ends are
5 dephosphorylated with calf intestinal phosphatase (CIP, 2 units in alkaline buffer, 30 minutes at 37°C), to prevent recircularization during the ligation step. The phosphatase is removed by extraction with phenol and chloroform, followed by precipitation with sodium acetate and ethanol on ice for 1 hour. The precipitated DNA is pelleted by high speed centrifugation and the ethanol is removed by aspiration, followed by a washing step with
10 70% ethanol to remove excess salts. The DNA pellet is air dried to completely remove any ethanol. The digested, phosphatased vector is then resuspended in TE buffer to 0.5 µg/µl. Approximately 0.1-0.5 µg of vector is incubated with a ten fold molar excess of the constructed variable region containing the sperm cell epitope in the CDR (modified variable region) with *T₄* ligase (1000 units) in appropriate buffer and incubated at 16°C for 12 hours.

15

7.3 BACTERIAL TRANSFORMATION

The ligation mixture containing the engineered variable region gene cloned into pUC19, is transformed into competent bacterial cells. Specifically, 50 µl of freshly prepared competent DH5-α cells are mixed with the ligation mixture of pUC19 and
20 modified variable region DNA and transferred to an electroporation cuvette (0.2 cm gap; Bio-Rad). Each cuvette is pulsed at 2.5 kV/200 ohm/25 µF in an electroporator (Bio-Rad Gene Pulser). Immediately thereafter, 1 ml of SOC media is added to each cuvette and cells are allowed to recover for 1 hour at 37°C in centrifuge tubes. An aliquot of cells from each transformation is removed, diluted 1:100, then 100 µl is plated onto LB plates with
25 ampicillin (Amp 40 µg/ml). The plates are then incubated at 37°C overnight and only cells containing a plasmid grow.

The plasmid DNA is analyzed after isolation from single colonies picked by sterile toothpick and grown up overnight in 3 ml LB/Amp in a sterile glass test tube, with constant shaking at 37°C. The plasmid DNA is isolated using Easy Prep columns
30 (Pharmacia Biotech) and suspended in 100 µl of TE buffer. To confirm the presence of insert, isolated plasmid DNA is digested with *Hinc II* and the digestion product is analyzed by 1.2% agarose gel electrophoresis in Tris-Acetate EDTA buffer (TAE). DNA is stained in the gel with ethidium bromide and visualized under UV light. The colonies that correspond to plasmids with insert are selected for further analysis.

35

7.4 DNA SEQUENCING

DNA sequencing is performed to verify the accuracy of the sequence in the cloned fragment. Sequencing across the pUC19 polylinker is done using the M13/pUC universal forward and universal reverse primers using the Sanger dideoxy chain termination procedure. The M13/pUC universal primers are readily found in biotechnology supply catalogues. Sequencing is performed on the ABI377 DNA sequencer, and sequence comparison is performed using standard computer alignment programs or visual inspection.

7.5 CLONING INTO THE V_H AND V_L CHAIN CONSTRUCTS

Once the sequence of the modified CDR has been confirmed, it is cut out of the pUC19 plasmid and ligated into either the heavy or light chain antibody expression vectors pMRRO10.1 or pGAMMA1, respectively (See Figures 6A and B). Alternatively, both the heavy and light chain genes are expressed on the same plasmid, and the modified CDR is ligated into either the heavy or light chain variable region as appropriate.

A complete antibody light chain has both a variable region and a constant region. A complete antibody heavy chain contains a variable region, a constant region, and a hinge region. The synthetic variable region genes of the invention are inserted into vectors containing appropriate constant regions. Engineered variable region genes with the sperm antigen epitope sequences are cloned into the pMRRO10.1 vector. The pMRRO10.1 vector contains a human kappa light chain constant region. Insertion of the engineered light chain variable region into this vector gives a complete light chain sequence. Alternatively, the engineered variable region gene with the sperm antigen sequence, of the heavy chain is inserted into the pGAMMA1 vector. The pGAMMA1 vector contains human and IgG1 constant region and hinge region sequences. Insertion of the engineered heavy chain variable region gene into this vector gave a complete heavy chain sequence.

In order to engineer a mammalian vector comprising both heavy chain and light chain genes, the complete light chain sequence and heavy chain sequence were inserted into a mammalian expression vector pNEPuDGV (Figure 6C; Bebbington, C., 1991, In METHODS: A Companion to Methods in Enzymology, 2:136-145). The resulting vector encodes both light chain and the heavy chain of the antibody.

7.6 TRANSFECTION OF EUKARYOTIC CELLS

The antibody expression plasmid, pNEPuDGV, is then transfected into a suitable host cell for expression of the antibody of interest. COS-7 (an African green monkey kidney cell line, CV-1, transformed with an origin defective SV40 virus), 293, or CHO cells are capable of being transfected and support expression of foreign proteins.

Transfection is performed by standard calcium phosphate precipitation (Sullivan et al., 1991, FEBS Lett. 285:120-123). Alternatively, cells may be transfected using lipid vesicles or electroporation. Transient or stable transfections are suitable depending on how much protein is expressed and harvested.

5

7.7 EXPRESSION AND PROTEIN ANALYSIS

Transfected cell supernatants are collected and analyzed for proper expression of anti-idiotypic antibodies. The antibodies are purified away from cell debris and growth media serum and also concentrated from the supernatant by binding the antibody Fc domain to a protein A or protein G column. The antibody is eluted from the column by low pH glycine and dialyzed against BSA and Tris buffer.

10

7.8 IN VIVO ANALYSIS OF ANTI-IDIOTYPE EFFICACY

To test the ability of the antibody to elicit an immune response or for a contraceptive effect, the antibody is injected into a mouse at a pharmaceutically significant dose range and serum samples are taken from the mice. The production of anti-idiotypic antibodies is confirmed by harvesting peripheral blood serum and performing ELISAs with the sperm antigen (or sperm), or western blots using the sperm antigen (or sperm) as target and the vaccinated mouse serum as probe.

15

ELISA involves capture of the samples and standards onto a 96 well plate coated with an anti-epitope antibody. Bound antibody is detected with a secondary antibody crosslinked to horse radish peroxidase (HRP) and the substrate tetramethylbenzidine (TMB) and specific to the kappa or lambda light chain of the mouse. Alternatively, western blots are performed using the anti-idiotypic as the target and probing it with anti-epitope antibodies.

20

25

Confirmation of production of anti-idiotypes in the mice is then followed by *in vivo* analysis to determine whether the mice are capable of conception. Control mice and test mice are mated in statistically significant groups and the number of pregnancies are monitored. Effective immunocontraceptive therapy will result in a significant reduction in the number of pregnancies.

30

Additionally, the induction of effective quantities of anti-idiotypic anti-bodies is also assayed for prevention of *in vitro* fertilization. Donor sperm is mixed *in vitro* with donor eggs in the presence or absence of test serum or negative control serum. The failure of sperm to fertilize the egg when test serum is added is a positive indication that the vaccine is effective.

35

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the
5 scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

10

15

20

25

30

35